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ARYLSULPHATASE AND THE HYDROLYSIS OF SOME STEROID SULPHATES IN DEVELOPING ORGANISM AND PLACENTA

BY

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Uuden Auran Oy:n kirjapaino

To my family

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PREFACE

This work was done at the Department of Physiology, University of Turku in 1956—1960.

I wish to thank first of all my teacher, Professor K. J. W. HARTIALA, who suggested this subject. He was always ready with his advice, never begrudging his time and gave me encouragement in many ways. To him go my very sincere thanks.

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Turku, March 1961.

Martti Pulkkinen

I

INTRODUCTION

Sulphatases are a group of esterases which split ester sulphates into sulphuric acid and the corresponding cationic group. The following general formula confirms this:

$$R-O-SO_3H + H_2O \rightleftharpoons R-OH + H_2SO_4$$

If we consider this reaction in another direction we are concerned with sulphate conjugation. Depending on the nature of R, a different sulphatase is needed for its decomposition and the following principal groups are generally obtained:

- arylsulphatase which hydrolyses aromatic and heterocyclic sulphuric acid esters;
- steroid sulphatase which is specific for the 3 β-sulphates of 5 α- and Δ^5-steroids;
- chondrosulphatase which hydrolyses the sulphates of chondroitin and mucoitin;
- glucosulphatase which hydrolyses the sulphuric acid esters of sugars;
- myrosulphatase which is the name given to the decomposing agent of a mustard oil glycoside. The substrate is sinigrin;
- alkylsulphatase which is so far known fairly theoretical only and hydrolyses alkyl sulphates, and
- aminosulphatase or sulph-amidase which participates in the hydrolysis of heparin.

The sulphatase enzyme group as a whole has been studied very little in human tissues (Bianchi 1955a, Dodgson, Spencer and Wynn 1956, Huggins and Smith 1949, Neuberg and Simon 1925, Pulkkinen 1957, 1960, Rosenfeld 1925, Rutenburg, Cohen and Seligman 1952, Rutenburg and Seligman 1956). Its investigation in various biological situations has been limited. In the literature the main point of focus has

been pure enzymology, and the material has often been a mollusc such as *Helix pomatia, Patella vulgata* or the African land snail *Otala punctata.* This is probably due in part to the speculative nature of our knowledge of the biological function of sulphatase. It is known that both glucuronate and sulphate detoxication are important in the removal of toxic agents and in some other metabolic occurrences. Closely associated with the latter are two of the most common sulphatases, which are capable at least *in vitro* and probably also *in vivo* of hydrolysing sulphate conjugates. The sulphate conjugates of steroids have a known significance since c. one-third of all steroids are excreted in the urine in compounds of this kind and for some steroids this form is probably more common than glucuronate. The blood, moreover, obviously has higher concentrations of sulphate than of glucuronate conjugates. Conjugated steroids are further said to be weaker biologically than free steroids. In order to throw light on the biological function of an enzyme it must be studied in different conditions. The enzyme concentrations of developing organisms in general and the localisation of the enzyme in certain organs are suitable physiological variables.

An endeavour has been made to modify the methods introduced earlier in the literature to suit the requirements of the present work. Both human and animal material has been used in the investigation itself. Arylsulphatase was analysed in different tissues to establish the enzyme level. The moment of the possible manifestation of sex differences was observed. The possibility of splitting steroid conjugates in the same conditions was studied with special reference to the rôle of the placenta. The activity of arylsulphatase was measured against p-nitrophenylsulphate, and of the steroid sulphates dehydroepiandosterone sulphate, estrone sulphate and androsterone sulphate were investigated.

II

REVIEW OF THE LITERATURE

Nomenclature and its development

The history of arylsulphatase dates back to 1911 when DERRIEN discovered in a gastropode, *Morex trunculus*, a factor which liberated indoxyl from indoxyl sulphate. Because of the indigo colour that originated, DERRIEN gave the enzyme the name purpurase. This observation failed for some years to attract much interest. NEUBERG and KURONO found the same factor again in 1923, in a commercial powder made from *Aspergillus oryzae*. This powder was Takadiastase or Takaminase. They began to use the name sulphatase. Later on, in 1932, NEUBERG and SIMON proposed the name phenolsulphatase for the enzyme to distinguish it from other sulphatases since specificity for the substrate had been observed. It was not until the 1950s that SPENCER above all suggested the name arylsulphatase, a closer approximation to reality (ROBINSON, SPENCER and WILLIAMS 1951). The enzyme is not uniform. To clarify the nomenclature, it may in fact be mentioned that enzyme type I of arylsulphatase is identical with fraction C and the insoluble enzyme, and that type II refers to fractions A and B, the soluble fraction.

The factor hydrolysing steroid sulphate conjugates was first called "alkyl sulphatase" and "steroid alcohol sulphatase" (STITCH and HALKERSTON 1953a). SAVARD, BAGNOLI and DORFMAN (1954) employed the term "neutral steroid sulphatase". The present name of the enzyme, "steroid sulphatase", was first introduced by ROY (1954) who, to emphasise further the specificity of the enzyme, suggested the name "3β-steroid sulphatase" (ROY 1956).

The following abbreviations are used in the present work:

NPS = p-nitrophenyl sulphate
NCS = nitrocatechol sulphate
DHAS = dehydroepiandrosterone sulphate
OS = estrone sulphate
AS = androsterone sulphate
U_w = enzyme unit calculated in terms of wet weight
U_N = enzyme unit calculated in terms of tissue nitrogen

A. Arylsulphatase

Distribution

The enzyme is very widely distributed in biological sources. Arylsulphatase of type II has been found among the higher plants in the vegetable kingdom (NEY and AMMON 1959). The most recent summary of the occurence of arylsulphatase in bacteria was published by VIRTANEN (1960). For some reason, the enzyme is not considered to be of great significance in the identification of bacteria.

In the animal kingdom, conchiferous animals and many worms contain arylsulphatase (NEY and AMMON 1959). The highest contents occurring in nature have been established in gastropods. *Helix pomatia,* in particular, has been used as a source of the enzyme (e.g. JARRIGE and HENRY 1952). Although its blood, liver, spawn and even lungs contain arylsulphatase (NEY and AMMON 1959), it is in the alimentary canal that the enzyme concentration is enormous. This finding has in fact inspired the view that the enzyme has some digestive function in these animals.

Arylsulphatase has been found in the liver of all mammalian species studied so far. It was first demonstrated in the animal kingdom by NEUBERG and SIMON in 1925. They studied the liver, kidney, brain and muscle of man, rabbit and guinea pig by using phenol ether sulphate as the substrate. The mammals studied include man, dog, rabbit, guinea pig, rat, mouse, ox and hamster.

Localisation in the different organs

Examination of the liver, kidney, brain and muscle of rabbit, guinea pig and man have revealed that the concentration decreases in the order of enumeration (NEUBERG and SIMON 1925). By sulphatase these authors meant the ability of crude homogenate to split phenol ether sulphate.

The following order of sulphatase activity was given by ROSENFELD (1925) for human organs: brain, kidney, liver, duodenum, adrenal gland, spleen, lung, small intestine, muscle and pancreas. He used potassium phenyl sulphate as the substrate. WOHLGEMUTH (1926) demonstrated sulphatase also in human skin. The following order of organs for rat, using the reaction between crude homogenate and NPS which thus involved primarily the determination of fraction C, was obtained: liver, adrenal gland, kidney, spleen, lymph node, lung,

thyroid gland, prostate, testis, heart, brain and, least skeletal muscle (HUGGINS and SMITH 1947). The hydrolysis of 6-benzyl-2-naphthyl sulphate by enzyme was studied in the tissues of 7 different mammalian species (RUTENBURG and SELIGMAN 1956). The tissues of hamster and rat showed the greatest sulphatase activity. The tissues of man, mouse, dog, guinea pig and rabbit had a much smaller sulphatase content. The liver was generally the tissue richest in enzyme, with kidney, pancreas and salivary gland next in order. These workers noted a moderate content in human tissue only in the liver, kidney and pancreas. The distribution of the enzyme in the organism has been studied earlier in this way.

The same questions were studied with better and more modern methods by DODGSON, SPENCER and WYNN (1956). They demonstrated all the three known fractions of arylsulphatase in human liver and analysed them also in some other tissues. They measured both type I and II and obtained the following order for type I: liver, pancreas, kidney, spleen, small intestine, lung, large intestine, brain and heart. The situation was roughly the same for type II, with small variations. The hydrolysis of NPS has been understood here as activity of type I, and for type II the substrate used was NCS. As was shown by ROY (1958), the ratio of the different sub-fractions of arylsulphatase varies in the different organs of various animal species. For instance, there were no demonstrable quantities of fraction C in guinea pig. In rat the greatest part of the sulphatase was of type B, and in the ox of type A. NEY and AMMON studied the different organs of *Helix pomatia* (1959) and found the enzyme in the liver, lung, spawn and alimentary canal.

Arylsulphatase in fluids and secreta

Arylsulphatase has been studied also in various body fluids and secreta. Cow's milk and colostrum and blood contain it (VAN KOETSVELD 1955). PANTLITSCHKO and KAISER (1952) were unable to demonstrate arylsulphatase activity in human urine. It can, however, be considered certain that there is some arylsulphatase in human urine. Thus, for instance HUGGINS and SMITH (1917) found both human serum and urine to contain some arylsulphatase. The urine contains 2 different arylsulphatases, A and B, which correspond to the same fractions found in human tissue (DODGSON and SPENCER 1956b). The electrophoretic mobility of these 2 fractions was identical with the mobility observed in arylsulphatase A and B of human liver and other tissues. Human urine and

serum normally show little activity against p-acetylphenyl sulphate and NPS (Dodgson and Spencer 1957a). In some conditions the urine shows a little greater activity towards these substrates, although it affects chiefly the sediment-depositing material. Both urine and serum show considerable enzyme activity towards NCS. The urine also contains a factor inhibiting pure A and B fractions. This factor is thermostable and dialysable and obviously involves sulphate and phosphate ions. Female urine contains an ostensibly greater quantity of sulphatase, but if the debris is centrifuged off the differences disappear. Urinary arylsulphatase can be concentrated c. 100-fold by acetone precipitation (Ammon and Ney 1957). The substrate used was NPS and NCS. Female urine showed a higher degree of sulphatase than male urine, and it increased further during menstruation. The maximum age for urinary arylsulphatase was c. 30 years. More detailed study of serum arylsulphatase (Dodgson and Spencer 1954) has shown that the activity is very low. Unlike fluoride and citrate, heparin and oxalate exerted no effect on the determinations. Determination with NPS was completed by a serum factor which hydrolyses NPS non-enzymatically in an alkaline milieu. The factor was thermostable and non-dialysable and had no effect on the sulphates of phenol, p-nitrophenol and phenolphthalein. Numerous sera under varying conditons were studied in the present work, but the result was meagre as regards enzymatic activity. According to Dzialoszynski (1957), normal urine had little activity when NCS was used as the substrate.

Localisation in different tissue and cell types

The enzyme has been localised in different cell types and parts of the organs by histological methods, too. The results achieved so far, however, have been open to criticism. Burton and Pearse (1952), for instance, criticised the investigations made and pointed out that the diffusion of 6-bromo-2-naphthol liberated from the 6-bromo-2-naphthyl sulphate used as substrate was considerable and that it combined with other tissue structures which were the actual site of the enzyme. Seligman, who used 6-bromo-2-naphthyl sulphate as substrate, was the first (1950) to give information concerning the possibilities of studying the distribution of the enzyme between different cell types. He noted that the enzyme was profuse in the periportal and pericentral parts of the liver. It was generally present in the cytoplasm of epithelial cells but not in the

nuclei, muscles or connective tissue. In man, the pancreas and especially the cytoplasms of the acinar cells stained heavily (RUTENBURG, COHEN and SELIGMAN 1952). The islets of Langerhans stood out from the surrounding acinar cells. The epithelial cells of the ducts gave a less intensive colour than the acinar cells. The activity of the adrenal gland was slight in the cortex. The most intensive colour appeared in the fascicular zone, and the amount of enzyme was smallest in the transitional zone. In the kidney, the enzyme was probably most profuse in the cortex. The cytoplasm of the epithelial cells stained intensely here, too, with the exception of the glomerulus. The proximal and distal parts of the tubuli were darker. MALMSTRÖM and GLICK (1952) studied the localisation of arylsulphatase in the adrenal gland. They pointed out themselves (GLICK, STECKLEIN and MALMSTRÖM 1955) that the results of their earlier investigation were misleading because they had neglected to take the blank values into consideration. In their later investigation they established the greatest degree of activity in the fascicular and reticular zone of the adrenal gland of rat, rabbit and dog, but were unable to establish any activity at all in any part of cow adrenal gland. They discussed in the same paper the adrenal glands of monkey and dog and the different histological zones of the adrenal gland of monkey. The results regarding activity were negative for the monkey. The corresponding dog organ showed relatively high activity in the fascicular zone.

Arylsulphatase was localised in the endometrium of the rat uterus in the epithelium of the glands (HAYASHI et al. 1955a). In another work (1955b) concerned with the histochemistry of enzymes in carcinoma of the mammary gland, uterus and prostate, these authors generally found more sulphatase in the epithelial cells than in the stroma. In rat adrenal gland arylsulphatase was localised primarily in the inner fasciculata and outer reticularis (GLICK and STECKLEIN 1956). Three summaries of the histochemistry of arylsulphatase are available in the literature (DODGSON and SPENCER 1957b, NEY 1959 and PEARSE 1960).

Intracellular localisation

The first to devote attention to the intracellular distribution of arylsulphatase was ROY (1953a). Fractions A and B isolated from ox liver were localised in the mitochondria. DODGSON, SPENCER and THOMAS (1953b) were of the opinion that the enzyme was indeed primarily

microsomal. Attention was later paid to the possibility that the different results obtained might have been due to the different substrates used. One research team had used p-acetylphenylsulphate, another NCS. Of the different types of enzyme, type I was localised in the microsomes and type II in the mitochondria (DODGSON and SPENCER 1957b). When the homogenate was fractionated in isotonic sucrose and the localisation of the different fractions of arylsulphatase type I was compared with acid phosphatase and glucose-6-phosphatase, the percentages illustrative of activity concurred well with the corresponding values of glucose-6-phosphatase (GIANETTO and VIALA 1955). Acid phosphatase on the other hand was largely localised in the light mitochondrial fraction which has relatively little activity towards p-acetylphenylsulphate. In another investigation, in 1955, VIALA and GIANETTO studied fractions A and B and observed their mitochondrial localisation. They found that it was fully analogous to that of acid phosphatase.

Fractions A and B are found to some extent in microsomes in addition to mitochondria (ROY 1958), but sulphatase C occurs only in microsomes.

Enzyme fractions

The complicated nature of arylsulphatase was considered in detail for the first time in 1953 when ROY established two fractions from an aqueous extract of an acetone powder of ox liver by fractional precipitation with acetone. He called these fractions A and B; they differed from one another in their optimal pH, optimal substrate concentration, activators and inhibitors. This activity was localised in the mitochondria. Fraction C was also isolated from ox liver (ROY 1956c). The corresponding fractions have been isolated from rat liver, too (DODGSON, SPENCER, and THOMAS 1955). Two different fractions, debris and supernatant, were obtained by centrifuging the homogenate obtained from the acetone powder of mammalian livers (DODGSON, SPENCER and THOMAS 1954). The former had a strong affinity for NPS and p-acetylphenyl sulphate which the latter did not split. The behaviour against NCS was the reverse. The results of the fractionating centrifugation were similar. Two different enzymes could be distinguished, one for its ready solubility after distribution of the mitochondria by acetone-drying, incubation in hyper- and hypotonic solutions or alternate freezing and thawing. The soluble rat enzyme, again, could be distinguished into two fractions corresponding to fractions A and B.

Human liver and many other tissues contain all three arylsulphatases (Dodgson, Spencer and Wynn 1956). The A and B parts were separated by paper electrophoresis from the soluble fraction. The B fraction moved more rapidly to the cathode. Attempts to separate completely the soluble and insoluble part of the enzyme from one another have failed. Human liver arylsulphatase B can be considerably purified by a procedure involving acetone fraction, treatment with protamine sulphate and adsorption of the enzyme on insintered-glass disks (Dodgson and Wynn 1958). All three fractions of ox liver have also been purified (Roy 1953b, 1954a, 1956c). It has not been possible yet, however, to obtain the enzyme in fully purified form.

Arylsulphatase A of ox liver has been purified 100-fold (Roy 1953b). There is also a method for the partial purification of sulphatase B (Roy 1954a). It has been shown in paper electrophoretic studies (Roy 1954a) that sulphatase A and B differ from esterase and phosphatase. Purified ox sulphatase C fraction has been observed to be very insoluble and it has been impossible to solubilise it (Roy 1956c). Fractions of human tissue have also been purified (Spencer and Wynn 1955).

Study of the affinity of various fractions for different substrates has shown that fractions A and B possess a great affinity for NCS (Dodgson, Spencer and Thomas 1955, Roy 1953b). Ox sulphatase A hydrolyses simpler arylsulphates in a much smaller degree than it hydrolyses NCS (Roy 1953b). Sulphatase C isolated from ox possesses a great affinity for NPS (Roy 1956b). The same applied to the preparations of sulphatase C isolated by Dodgson. The debris fraction in the liver of mammals split NPS and p-acetylphenylsulphate, but the supernatant did not. Its reaction with NCS was the opposite. The insoluble enzyme has a high affinity for NPS and acetylphenylsulphate, while the soluble enzyme has a low affinity for these substrates but a higher activity towards NCS (Dodgson, Spencer and Thomas 1955). The NPS curves of fraction A purified by means of paper electrophoresis show that this enzyme has a low affinity for this substrate and that its kinetic behaviour differs markedly from that of the insoluble enzyme. The NPS activity of enzyme B was almost negligible (Dodgson, Spencer and Wynn 1956a). Human liver arylsulphatase B was active for 2-hydroxy-5-nitrophenylsulphate (NCS) but showed little activity towards potassium phenyl sulphate and its monosubstituted derivates; there was appreciable activity towards disubstituted derivates (Dodgson and Wynn 1958).

Optimum determination conditions

The optimum enzymological conditions for the determination of arylsulphatase vary with the substrate or buffer used, the source of the enzyme, and the activating or inhibiting ions involved; in certain cases even the enzyme concentration used influences the reaction conditions, e.g. fraction A. (Dodgson and Spencer 1956a). The optimum conditions for assay of arylsulphatases from various sources have been reviewed by Dodgson and Spencer (1957a). They compiled two tables for the changing of these different variables in relation to one another. The present author has concentrated here on earlier findings concerning the optimum conditions for this investigation. Huggins and Smith (1947) established 6.12 as the optimum pH of rat liver at 37°C with 0.5 N acetate buffer and 0.015 M as the optimum substrate concentration when p-nitrophenyl sulphate was used. Using an unknown substrate concentration, Abbot and East (1949) established 6.6 as the optimum pH for rat liver and kidney and 50°C as a suitable incubation temperature. The arylsulphatase activity of the adrenal gland of rat towards NPS was measured in acetate buffer, pH 5.8, using a substrate concentration of 0.0005 M (Glick, Stecklein and Malmström 1955). These earlier investigations were performed with whole homogenate. For purified rat arylsulphatase C, using NPS as the substrate in 0.5 M acetate buffer, the optima are: pH 7.0 and substrate concentration 0.006 M (Dodgson, Spencer and Thomas 1955). For fraction C isolated from human tissues the optima are 7.3 and 0.008 (Dodgson, Spencer and Wynn 1956a). For rat arylsulphatase A and B with NPS the optima are 5.8—0.12 and 6.2—0.12, and for human arylsulphatase A 6.2 and 0.07.

Inhibition and activation of the enzyme

By may of a general statement it can be said that arylsulphatase of type I is inhibited by cyanide but hardly affected by phosphate or sulphate. Type II, on the other hand, is unaffected by cyanide but is strongly inhibited by phosphate and sulphate (Dodgson and Spencer 1957a). Inorganic phosphate is identified as an endogenous, non-competitive inhibitor of type II (Maengwyn-Davies and Friedenwald 1954). Substantially all of the inorganic phosphate is in the undialysed preparation. The activity of freshly thawed, but undialysed preparations

was found to be markly less than that of similar preparations after exhaustive dialysis against running tap water. Activation by some of the heavy metals may presumably be due to removal of the inorganic phosphate left in the preparation after dialysis. This endogenous inhibition lends interest also to the ratio between acid phosphatase and arylsulphatase. Endogenous inhibition attracted the attention of RUTENBURG and SELIGMAN (1956) also. The high endogenous phosphate content of rat kidney that they obtained produced an inhibition of enzymatic activity in acetate buffer which was greater than that noted with liver.

Several workers have found quite a number of different general biological enzyme inhibitors to be suitable also for arylsulphatase (DODGSON and SPENCER 1953b, DZIALOSZYNSKI 1947, 1950, HOMMERBERG 1921, ROBINSON et al. 1952, SELIGMAN, CHAUNCEY and NACHLAS 1951, TORDA 1943). However, as far as the older investigations are concerned there can be no certainty about which fraction was measured in each case. The inhibition and activation of different arylsulphatase types by pure enzyme preparation was studied by the research teams of DODGSON and ROY (DODGSON and POWELL 1959, DODGSON and SPENCER 1957a, DODGSON, SPENCER and WYNN 1956, and ROY 1954a, 1955, 1956b).

Solubility of arylsulphatase

Two different arylsulphatase types differ in their solubility. Fractions A and B have been demonstrated in an aqueous solution obtained from an acetone powder of ox liver. These fractions were water-soluble (ROY 1953 b, 1954 a). Sulphatase C, on the other hand, could not be solubilised with a wide pH range of buffer and hyper- or hypotonic saline solutions through alternate freezing and thawing or mechanical disruption (SPENCER et al. 1955). Trypsin solubilised a part of arylsulphatase but caused a decrease in activity. The enzyme could be solubilised by using either a cationic or non-ionic surface-active substance, resulting increased arylsulphatase activity on account of the greater enzyme dispersion (DODGSON et al. 1957b). Solubility and activation effect seem to be associated with the formation of micelle together with the solvent. Anionic agents solubilised the enzyme, but caused inhibition of activity of arylsulphatase. Fractions A and B are readily solubilised from the mammalian liver after rupture of the mitochondria of the liver cells (VIALA and GIANETTO 1955). In this insoluble condition, arylsulphatase

A and B show only a part of their total activity and the granules with which they bind become irreversibly activated when a method is used that dissolves the enzyme: treatment with hypotonic sucrose solution or corresponding saline solution, mechanical disruption, freezing and thawing and incubation at 37°C together with isotonic sucrose solution. Although sulphatase A and B are localised mainly in the mitochondrial and microsomal fraction, they cannot be completely separated from them with water.

Mechanism of action

Something is known of the details of the structure and mechanism of action of the enzyme. Dodgson, Spencer and Williams (1956) studied the changes in the affinity between the substrate and the enzyme by binding various substituents with the benzene ring. They used an arylsulphatase obtained from *Alcaligenes metalcaligenes*. The inclusion of electrophilic groups such as the nitro-group increases the affinity between the enzyme and the substrate by raising the positive charge of the S-atom of the residual sulphate. Nucleophilic substitutes have the contrary effect. These authors have published a table showing how the Michaelis constant K_m and V_{max} with phenolsulphate as the basis change when different substituents are included in the phenol ring. For NPS it is $10^4 \cdot K_m$ 4.75. There must be an intensive "electron-withdrawing" effect on the sulphate group of arylsulphate before it can be hydrolysed by arylsulphatase to any considerable extent. Dodgson, Spenger and Williams introduced also a hypothetical formula for the mechanism of the enzyme action.

Roy studied a number of inhibitors and came to the conclusion that arylsulphatase C is an SH enzyme (1956b). The activity of the A fraction of ox liver is not directly proportional to the concentration. It seems that this effect can be explained as the polymerisation of enzyme molecules, producing a complex which is more active than the non-polymerised molecule (Roy 1954b). Later he wrote that this hypothesis was not correct (Roy 1960). Sulphatase A of ox liver has, like C, been classified as probably an SH enzyme (Roy 1956b), but for B the matter is not clear. There is some indication as to the nature of the ionising group that brings about the formation of the enzyme-substrate complex and the cleavage in each individual case. This problem has been investigated by Dodgson and Wynn (1958). There

are two possible ways in which a compound of type $Ar \cdot O \cdot SO_2 \cdot O$ splits into $Ar \cdot OH$ and $HO \cdot SO_3$. The bond may break either between Ar-O or the O—S link may split. In reality, it is the latter alternative that occurs (SPENCER 1958). SPENCER hydrolysed arylhydrogen sulphates with acid, alkalis and enzymes in a solution containing $H_2\ ^{18}O$. The liberated sulphate was isolated and its ^{18}O activity determined. The sulphate part had radioactivity, i.e. the O—S linkage had split irrespective of the hydrolysing agent used.

The possible rôle of arylsulphatase in conjugation involves an altogether different effect on the mechanism of action. If arylsulphatases act as transferases they catalyse the transfer of the sulphate rather than of the phenolic group (SPENCER 1957). The sulphated polysaccharide of the mucous gland of *Charonia lampas* (charonin sulphate) can be sulphated with a system which contains arylsulphate, arylsulphatase, carbohydrate acceptor and at least one unknown factor (SUZUKI, TAKAHASHI and EGAMI 1957). This transsulphation is inhibited by phosphate and fluoride in the same manner as the hydrolysis of arylsulphates is inhibited by arylsulphatase. Judging by this work of SUZUKI, TAKAHASHI and EGAMI, it seems that arylsulphatase really does participate itself in a reaction of this kind. What seems to be involved here is the transfer of sulphate from one sulphate ester to another, a movement which is directly synthesised from PAPS, adenosine-3'-phosphate-5'-phosphosulphate. An example is the synthesis of ^{35}S charonin sulphate and glucose-6-^{35}S — SO_4 from charonin sulphate and NPS under the influence of an acetone-dried preparation obtained from the mucous gland of *Charonia lampas*. No transfer occurred when the p-nitrophenyl ^{35}S-sulphate was replaced by ^{35}S-$K_2\ SO_4$.

Arylsulphatase in different physiological conditions

Some investigations have touched upon the arylsulphatase content of the organism as a function of age. Urinary excretion of arylsulphatase is fairly small in children, increases later, reaches its maximum at the age of 30—40 and falls again with the approach of old age (AMMON and NEY 1957). The phenolsulphatase content of the aorta and the pulmonary artery decreases with age (KIRK and DYRBYE 1956) and shows fairly good correlation with arteriosclerotic changes. Arylsulphatase C has been found to be much more profuse in the liver of male than of

female rats. The mouse strain studied, on the other hand, revealed no sex difference. The activity of sulphatase A and B is increased in proliferating tissue (Roy 1958). It is extraordinary that castration has no effect on the sex difference. The activity of sulphatase A and B was very high in four young rats, while the activity of both sulphatase C and steroid sulphatase was slightly lower than normal. Reference has been made to the sex difference in urine, and it would seem that female urine contains a greater amount of arylsulphatase C (Dodgson and Spencer 1957 a). This, however, is due to the more profuse sediment. If the sediment is centrifuged off carefully, female urine has as much enzyme as male urine. The same applies probably also to certain observations of a higher arylsulphatase concentration in the urine during menstruation (Ammon and Ney 1957). Hayashi et al. (1955) noticed, however, that castration had a lowering effect on the arylsulphatase content of the rat uterus. The cyclical changes of the enzyme in the uterine wall were definite. The arylsulphatase in the apical cytoplasm in the proestrus increased on entering early estrus and changed to basal cytoplasm in the actual estrus (Hayashi et al. 1957). The time of the day has also been considered to influence the sulphatase concentration. In the urine, maximum values were found at noon. Serum gave the same result (Boyland, Wallace and Williams 1955). The effect of nutritional factors on sulphatase activity has also been discussed (Dodgson, Lewis and Spencer 1953).

Rat given 50 IU of estrogen showed the highest arylsulphatase values 4 days later in the endometrium and especially in the epithelium of the uterine lumen (Hayashi et al. 1955). Only a weak positive reaction remained 6 months later, and the "increasing stadium" in such situations was shorter for arylsulphatase than for β-glucuronidase. The phenol sulphatase of guinea pig liver rose when the animal was given estrogen (Bianchi 1955a). Experiments have also been conducted concerning the effect of ACTH, DOCA and cortisone on the enzyme content, and the stress conditions of turpentine abscess or cold were studied at the same time. No changes were observed histologically in the distribution of phenol sulphatase activity when the results were calculated in terms of the protein-nitrogen content (Glick and Stecklein 1956). A notable fall was observed in the activity in the fascicular and reticular zone 6 days after hypophysectomy and this effect was of almost the same order after 30 days.

The present author has published previously a preliminary report on

the occurrence of arylsulphatase in man during the fetal period (PULKKINEN 1957). Rat seemed to have a tendency during its fetal development to rising arylsulphatase concentrations in the liver (HARTIALA et al. 1958), no differences were observed after the livers were cultured on a fungus.

Origination of substrate

The mode of origination of the substrate for arylsulphatase does not actually come into the present work. A possibility that must be considered, however, is that arylsulphatase might perhaps be present in the chain of synthesis in certain of the special cases cited. The main principles of the present view of sulphate conjugation were introduced by FRITZ LIPMANN in 1958. The first step is the formation of active sulphate and the enzymes associated with it are common in conjugates of various types.

The reaction between adenosintriphosphate and sulphate produces through the agency of sulphurylase and ATP-kinase an active sulphate, adenosine-3'-phosphate-5'-phosphosulphate. From here the sulphate is transferred through the agency of a more or less specific enzyme, sulphokinase, to steroid, phenol, etc.

Phenols have a common phenolsulphokinase, estrone has its own and there is again a common one for 3 β-hydroxysteroids. Chondroitin sulphuric acid and the cerebroid sulphate of the brains thus also obtain their sulphate group in the same way.

If the sulphatase participates in this mechanism it obviously transfers the sulphate from one ester to another, and the sulphate ester is synthesised direct from adenosine-3'-phosphate-5'-phosphosulphate. Substrates for sulphatase originating in this way can be found in the organism.

B. Steroid sulphatase

Hydrolysing of steroid sulphates in the organism

Steroid sulphatase is highly specific, capable of hydrolysing only a small part of all steroid sulphates. If the steroid has an aromatic ring, as is the case with estrone sulphate, steroid is liberated by ordinary arylsulphatase. It has not been possible to split 3 α-steroids, e.g. androsterone sulphate, by means of biological materials.

The information on steroid sulphatase in mammals has been limited so far to its occurrence in the liver. It has been demonstrated in guinea pig also in the spleen and the adrenal gland (GIBIAN and BRATFISH 1956). It has not been established prior to the present work in an organ so important for the steroid balance during pregnancy as the placenta. The present author published a preliminary communication on the subject elsewhere (PULKKINEN 1960).

Distribution, localisation and properties

Steroid sulphatase was first found in the intestinal fluid of *Helix pomatia* (HENRY and THEVENET 1952). The steroid sulphatase in mammals was demonstrated by GIBIAN and BRATFISH (1956). They used rat and cow liver as the source of the enzyme. ROY, in his investigation of a later date, worked with ox and rat liver (1957). Plants, bacteria and fungi obviously do not contain steroid sulphatase (NEY and AMMON 1959). The liver of fish and birds contains this enzyme. The same investigation established steroid sulphatatic activity in the adrenal gland, liver and spleen of guinea pig. It has not been possible to establish it in human urine.

Steroid sulphatase is localised intracellularly in the mitochondrial fraction (ROY 1957).

Steroid sulphatase has been separated electrophoretically from other sulphatases (ROY 1956a). The optima for its determination in *Patella vulgata* enzyme were as follows: pH 4.5, substrate concentration 0.2 mM. The results show a good fifth of the LINEWEAVER and BURK equations and gave a value of 0.04 mM DHAS for K_m. This substrate concentration has also been used with an enzyme derived from mammals (GIBIAN and BRATFISH 1956 and ROY 1957); the optimum pH, however, was higher than for *Patella* enzyme. GIBIAN and BRATFISH determined rat liver steroid sulphatase activity at pH 7.3—7.5, using 0.5 M triethanol ammonium acetate buffer. ROY used pH 7.8 with ox liver in TRIS buffer.

A table was published by ROY (1954b) for the inhibition of steroid sulphatase. It showed phosphate, fluoride and sulphate as the strongest common inhibitors. The same observation was confirmed by SAVARD, BAGNOLI and DORFMAN (1954) and by GIBIAN and BRATFISH (1956). This enzyme is water-soluble.

It has been emphasised that steroid sulphatase is highly specific. It hydrolyses only the 3 β-sulphates of the 5 α- and Δ^5 -series. Estrone sulphate e.g. is thus hydrolysed under the influence of arylsulphatase.

Several investigators have used steroid sulphatase for splitting the urinary sulphate conjugates (COHEN and BATES 1949, HENRY and THEVENET 1952, HENRY, THEVENET and JARRIGE 1952, JAYLE and BAULIEU 1954, STITCH and HALKERSTON 1953a and b, and STITCH, HALKERSTON and HILLMAN 1956). In general, no attention has been paid, however, to the relatively narrow specificity of the enzyme, and the value of steroid sulphatase as a substitute for acid hydrolysis is questionable. It may be helpful in some special cases if the structure of the steroid must be kept completely unchanged. Hydrolysis of the neutral 17-oxosteroids of the urine with enzyme or acid revealed in chromatography that there were differences in the distribution of the chromatographic fractions (STITCH and HALKERSTON 1956). GIBIAN and BRATFISH (1956) stated concerning the stability of the enzyme that c. 50 per cent of the activity remained in their acetone powder after 3 months' storage.

There is a mention from the field of physiology that rat liver displays sex differences, and male rats have been found to possess more profuse steroid sulphatase than female rats but a smaller sulphate conjugation ability (ROY 1958).

III

THE PRESENT INVESTIGATION

THE PROBLEM

The purpose of the present investigation was to study the following problems:

(1) Control of the p-nitrophenyl sulphate method and its application in the present study.

(2) The arylsulphatase content of different organs during fetal development in man and in rat and differences between the organs in this respect.

(3) The arylsulphatase activity of the different organs of rat during extrauterine development. Attention was also paid to the differences between the sexes and the time they appeared.

(4) The hydrolysis of arylsulphate conjugates in the placenta.

(5) The hydrolysis of steroid sulphate conjugates in the developing organism of man and rat during intrauterine life.

(6) The ability of growing rat to hydrolyse steroid sulphates.

(7) The placenta as the producer of free steroids from their sulphate conjugates, with special reference to the existence of specific steroid sulphatase.

(8) The ability of hydrolysing 3α-steroid sulphate by various tissues.

MATERIAL

A. Arylsulphatase

The material was divided into two principal groups: human and animal.

The human series consisted of 71 fetuses whose crown-heel length ranged from 2.5 to 33 cm. Twenty seven different tissues and secreta of these fetuses were analysed for arylsulphatase content as far as size permitted. The material was collected from *sectio minor* operations

Table I. Distribution of the rat material by age and sex for arylsulphatase determination.

Sex	Age or size	Number
—	fetus of under 4 g	49
—	fetus of over 4 g	15
—	newborn	46
male	1 month	20
female	1 month	16
male	adult	30
female	adult	26
		Total 202

for legal abortion at the Women's Hospital, University of Turku, the Maternity Hospital of the City of Turku and Turku Nursing Home. Three cases of diabetes comprised the only endocrinological indications for interruption of pregnancy. Fifty five placentas of fetuses of different ages were collected in the same connection. Their enzyme activity was analysed on both the maternal and fetal side. A further 29 placentas obtained from normal vaginal deliveries were collected from the Women's Hospital, University of Turku.

The test animal used was the Wistar rat strain (inbred in this laboratory during the last 5 years). The animal material consisted of 65 rat fetuses of different age from 24 mothers, 46 newborn (under 24 hours of age), 36 month old and 56 adult rats aged 3 ½ months, i.e. 202 animals in all. The sex distribution of the main groups was as follows: 16 female rats aged 1 month, 20 male rats of the same age and 26 adult female and 30 adult male rats. The distribution is shown in Table I. The rats were kept throughout the experiment on a fairly standard diet consisting of mixed foodtype. This has been found to be a suitable fare, judging chiefly by good reproductive ability and coat of hair and lack of other deficiency signs. The material was collected in different seasons, simultaneously from all the different groups. The fetuses were weighed, and weight was used as the criterion of the stage of development. It is difficult to determine the duration of the pregnancy of a rat: the time of fertilisation is always somewhat uncertain.

Arylsulphatase activity was determined from the liver, kidney and intestine.

Table II. Distribution by age and sex of rats used for determination of hydrolysis of DHAS and OS.

Age	DHAS			OS		
	Male	Female	Total	Male	Female	Total
fetus	—	—	23	—	—	29
under 24 hours ..	12	5	17	12	4	16
1 month	14	14	28	12	15	27
adult	15	15	30	15	13	28
Total	41	34	98	39	32	103

B. Steroid sulphates

The material was divided into two parts, human and animal. Table II shows the size of the groups of rat material. A total of 102 animals were analysed for hydrolysis of dehydroepiandrosterone and estrone sulphate to accertain the age development.

The rats were the same as and kept in identical conditions with those in the part of the work concerned with arylsulphatase.

Six human fetuses were analysed; they were collected in the same way as for arylsulphatase determinations.

The placentas obtained from spontaneous human deliveries, 29 in all, were analysed from the maternal and the fetal side for both steroid sulphates.

Random samples were collected from rats, human placentas, parts of human digestive tract obtained at operations and from fresh human fetuses for study of the hydrolysis of androsterone sulphate. The samples were treated in the same way as for the arylsulphatase determinations.

METHOD

Treatment of the samples

The fetuses were placed in a +4° C refrigerator immediately after the operation. The different organs were prepared as carefully as possible in a room of the same temperature and the pieces of tissue selected were weighed on an analytical balance (Sartorius Selecta, accuracy 0.1 mg).

The rats were killed by a blow on the neck. The pieces of tissue were weighed immediately as above, and kept during the manipulation at +4° C.

From the placentas, sample sections were cut from the maternal side at a site with no distinct calcification. A section taken from directly below the membranes was called the fetal side. The blood was drained from the samples by blotting paper and the samples were treated in the routine way.

A. Arylsulphatase

Main principles of the determination

The earliest investigators used the gravimetric method to establish enzyme activity. The unhydrolysed phenol ether sulphuric acid was hydrolysed by hydrochloric acid, and the liberated sulphate was determined as the sulphate of barium or benzidine by weighing (Neuberg 1923). Measurement of the liberated phenol was also used. Dodgson and Spencer have given an extensive summary of the different methods employed (1957a). They can be divided into three principal groups according to the component of the reaction $R-O-SO_3H + H_2O \rightarrow R \cdot OH + H_2SO_4$ that is to be measured. It is thus possible to measure

(a) $R \cdot OH$
(b) H_2SO_4
(c) $R-O-SO_3H$ which failed to hydrolyse.

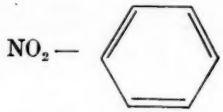
The first of these methods is the one most commonly chosen. Phenol or another similar agent can generally be most easily determined spectrophotometrically, and this in fact is the most common procedure. The commonest substrates of arylsulphatase are NPS and acetylphenyl sulphate (for type I) and NCS, i.e. 2-hydroxy-5-nitrocatechol sulphate (for type II), and hence it is often necessary to determine the corresponding phenols. The second alternative has certain advantages. It can be employed for the subtrates of all sulphatases. Liberated sulphate can be determined in various ways.

The third alternative is to measure the substrate that has failed to hydrolyse. The earlier method (Neuberg and Wagner 1927) of hydrolysis and then determining the liberated sulphate gravimetrically involves prolonging the incubation time because there has to be a rela-

tively great difference between the enzymatically hydrolysed and unhydrolysed substrate (Roy 1956a).

The method used here was based on the determination of the liberated phenol.

Arylsulphatase was determined by Huggins-Smith's method (1947) using NPS as substrate. The method was modified for the purposes of the present work.



Potassium salt of p-nitrophenylsulphate

Reagents

(1) Acetate buffer. 68.08 g of $CH_3 \cdot COONa + 3\,H_2O$ (Merck, *p.A.*) was weighed ad 1 l of distilled water and allowed to dissolve overnight. The pH was adjusted to the desired level with 0.5 N acetic acid. Four solutions were prepared: pH 7.3, 7.1, 7.0 and 5.8. The pH was controlled by glass electrodes in a Beckman pH meter. The pH of the solutions were checked occasionally.

(2) Substrate, p-nitrophenylsulphate (Sigma Chemical Co.). The reagent was kept crystalline in an exsiccator at + 4°C and only the amount required daily was dissolved in water. The reagent keeps fairly well in crystalline form; after several months the blank values may rise, in which case a red colour is seen in the p-nitrophenylsulphate. When this happened the substrate was discarded. For rat tissues, 1.285 mg of NPS was dissolved in 1 ml of distilled water. For human tissues the corresponding amount was 6.425 mg/ml.

(3) Sodium hydroxide (Merck *p.A.*). Sodium hydroxide of two different potencies was used: 0.5 N and 0.63 N.

(4) 10 per cent zinc sulphate (Merck *p.A.*), $ZnSO_4 + 3\,H_2O$.

Homogenates and the reaction conditions

The weighed pieces of tissue were homogenised by Potter-Elvehjem's (1936) glass homogeniser in a refrigated room at + 4°C until the homogenate looked even. The most important variables in the reaction conditions are shown in Table III.

Table III. The reaction milieu in the determination of arylsulphatase of some tissues.

	Tissue	Homogenate %	Incubation time, hours	pH	Substrate concentration	Normality of NaOH
Rat	liver	1	3	7.0	0.001	0.5
	kidney	0.5	„	5.8	„	0.63
	intestine	1	„	7.0	„	0.5
Man	liver	0.2	1	7.3	0.005	0.5
	kidney	1	„	7.1	„	„
	intestines	„	„	7.3	„	„
	pancreas	„	„	„	„	„
	placenta	„	„	„	„	„
	brains	3	3	„	„	„
	lungs	„	„	7.1	„	„

The wet-weight percentage of the homogenates was 1, except for rat kidney for which it was 0.5, for human fetus liver 0.2 and for brains and lungs 3. The rat kidney percentage was changed because, due to the small size of the fetal kidneys, it was impossible to obtain a 1 per cent homogenate from the fetus material; hence 0.5 per cent was used throughout the work. For human fetus the homogenates were changed to allow better determination of activity. Three-hour incubation was considered more suitable for rat tissues. For human fetuses 1 hour was sufficient for liver, kidney, intestines, pancreas and placenta, for other tissues the incubation time was 3 hours. The substrate concentration used was 0.001 for rat tissues and 0.005 M for human tissues; both were below the optimum. Because of the low pH of the buffer it was necessary to use a stronger base for precipitation of rat kidney in order to achieve full intensity of colour.

Determination of activity

Into 1.5 ml of acetate buffer was pipetted 0.5 ml of homogenate at the optimum pH of each sample (Table III). Ordinary test tubes were used. The tubes were pre-incubated for 3 min. in a water bath at + 37°C. The reaction was then started by adding 0.5 ml of NPS solution into the tubes to a final molarity of 0.001 in the rat analyses and of 0.005 in the analyses of human fetuses and placentas. After the reaction time set for

each sample (1 hour or 3 hours), 0.8 ml of sodium hydroxide was added to the tubes to stop the reaction. Into one tube at a time was added 0.5 ml of 10 per cent zinc sulphate and the tubes were shaken carefully. Immediately after this the tubes were filtered by suction through Whatman No. 40 filter paper (Ø 7 cm). The p-nitrophenol colour that originated was measured by Beckman-DU-spectrophotometer at wave length 402 mμ in normal cuvettes against distilled water. All determinations were made in duplicate. The procedure was the same in the control determinations except that the homogenate was added to the test tube immediately before pipetting the base. This was done with the various tissue types at each determination.

Determination of tissue nitrogen

The tissue nitrogen of the homogenates was determined by the micro-Kjeldahl method (see e. g. HAWK, OSER and SUMMERSON 1954) using K_2SO_4-$CaSO_4$ during the evaporation of the water and adding 30 per cent hydrogen peroxide. The ammonia was distilled in boric acid and titrated with sulphuric acid to the colour-change point of bromcresol-green methylred mixture as indicator. One ml of the 0.1 N sulphuric acid corresponded to 0.14 mg of nitrogen. The method was accurate to 0.1—1 mg of nitrogen.

The enzyme unit and its calculation

The unit of arylsulphatase was calculated from the following formula:

$$\text{Number of units } U_w = \frac{\mu\text{g of liberated p-nitrophenol}}{100 \text{ mg w/w} \cdot \text{hours}} \text{ or here}$$

$$\frac{\mu\text{g of liberated p-nitrophenol} \cdot 20}{\% \cdot \text{hour}}$$

The results were also calculated per tissue nitrogen.

$$\text{Number of units } U_N = \frac{\mu\text{g of liberated p-nitrophenol}}{\text{mg nitrogen} \cdot \text{hour}} \text{ or here}$$

$$\frac{\mu\text{g of liberated p-nitrophenol} \cdot 2}{\text{mg nitrogen} \cdot \% \cdot \text{hour}}$$

The total of μg of liberated p-nitrophenol was obtained by subtracting from the Beckman reading the blank value and multiplying the result by 0.0492. This figure was obtained from the calibration curve which was linear in the zone studied.

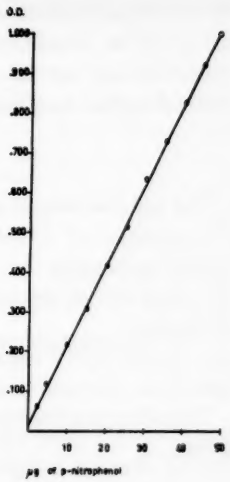


Fig. 1. Calibration curves for p-nitrophenol.

Re-crystallised p-nitrophenol (Fluka) was employed in the calibration. It was performed in exactly the same way as the procedure used in enzyme determinations, substituting p-nitrophenol for the substrate. Similar calibration curves were obtained with 1 per cent rat liver homogenate or water instead. Six parallel determinations were made for both at 5 μg intervals over the range 2.5—50 μg.

B. Hydrolysis of steroid sulphates

Steroid sulphatase can also be determined in three different ways: either liberated sulphate or steroid is determined and, in the third method, the residual substrate. The first method has not been used, but now when there is a better method than before of determining inorganic sulphate by means of benzidine or barium chloranilate (HÄKKINEN 1959, 1960) this method may be the best. Liberated steroid can be determined in the routine way by the ZIMMERMAN technique (ZIMMERMAN 1936). This method was used e.g. by GIBIAN and BRATFISH (1956). The technique for the determination of residual substrate is also relatively simple. Steroid sulphate and methylene blue combine to form a complex which can be extracted in chloroform. This complex is not formed by the free steroid. The method is applicable to all steroid sulphates (ROY 1956a).

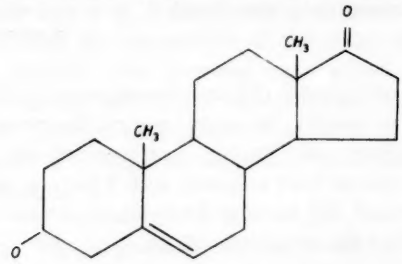
The splitting of steroid sulphates was studied here by determining the

unhydrolysed steroid sulphate as a methylene blue complex which was extracted in chloroform (Roy 1954b) and using the enzyme reaction conditions set out in the literature for rat liver (Roy 1957). Minor methodological modifications were made.

Choice of substrate

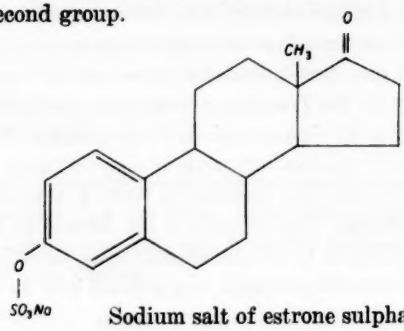
The purpose was to analyse 3 basic types of steroid sulphate:
(1) sulphate of 3 β-steroid, which would be a specific substrate for steroid sulphatase.
(2) Sulphate of aromatic steroid, which would be split by common aryl-sulphatase.
(3) Sulphate of 3 α-steroid, which hardly occurs in nature and which it has not so far been possible to split biologically.

Dehydroepiandrosteron sulphate, DHAS, was selected for the first group.



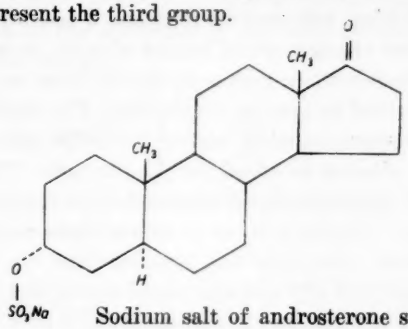
Sodium salt of dehydroepiandrosteron sulphate

DHAS has been used almost exclusively in the literature and it is hydrolysed well by specific steroid sulphatase. Estrone sulphate (OS), which was also used in the present work, has generally been used to represent the second group.



Sodium salt of estrone sulphate

Androsterone sulphate is a typical sulphate of 3 α-steroid and it has been used to represent the third group.



Sodium salt of androsterone sulphate

Reagents

(1) 0.5 M TRIS, i.e. 2-amino-2-hydroxymethylpropane-1:3-diol-acetic acid buffer (Sigma Co.). 6.05 g of TRIS was weighed to make 100 ml of the buffer. Using concentrated glacial acetic acid, the solution was adjusted to pH 7.8.

(2) 0.4 mM steroid sulphate. The steroid sulphates used were sulphates of dehydroepiandrosterone, estrone and androsterone (Schering Ag.). DHAS and AS were crystalline in ampules, but OS was dissolved in triethanolammoniumacetate as a concentrate. 1.6 mg of DHAS was weighed ad 10 ml of distilled water, and the same amount of AS. From this 4 mM solution, 0.4 mM was diluted with distilled water for each day. A stock solution of 4 mM was also made of OS by diluting the estrone sulphate solution with distilled water.

(3) Chloroform (Merck *p.A.*); *pro analysi* chloroform was used undistilled.

(4) Methylene blue reagent. 125 mg of methylene blue (Methylenblau Geigy), 25 g of anhydrous Na_2SO_4 and 10 ml of concentrated sulphuric acid (Merck *p.A.)* was dissolved in a small amount of water and diluted ad 1000 ml.

(5) Alcohol. Technical 96 per cent ethanol (Rajamäki factories) was diluted with distilled water to 75 per cent or used as such.

Determination of activity

Into round-bottomed centrifuge tubes was pipetted 0.2 ml of TRIS buffer and 0.4 ml of aqueous solution of steroid sulphate. After preincubation for 5 min. at + 37°C, 0.2 ml of homogenate was added

to the tubes. The incubation time was 6 hours for DHAS and 3 hours for OS when rats were in question, 3 hours for DHAS and 1 hour for OS when homogenates of human placenta were studied, and 20 hours for all steroids when tissues of human fetus were analysed. The tubes were stoppered to prevent evaporation. The reaction was stopped and the proteins precipitated by adding 5 ml of 96 per cent ethanol, and the tubes were allowed to stand for 15—20 min. The sediment was centrifuged and the clear liquid decanted. Five ml of the clear liquid was pipetted into Hagedorn tubes in which it was evaporated dry in a boiling water bath. Each tube was removed from the water bath after it had been evaporated dry and was cooled in running cold water. After the cooling, 2 ml of methylene blue reagent and 5 ml of chloroform were added to the tubes. They were shaken for 30 sec. by the lateral shaking method. Because an emulsion sometimes forms, the samples were always centrifuged. The topmost layer of methylene blue was sucked away as carefully as possible by Pasteur pipette. Two ml of the chloroform phase was pipetted into 10 ml of 75 per cent ethanol, and the tubes were shaken carefully. The intensity of the colour was measured at wave lenght of 663 mμ against a blank using a light cell suitable for this wave length. All the analyses were made in duplicate.

The control for the steroid sulphate that failed to hydrolyse was always performed again as a duplicate or triplicate determination. It was made in exactly the same way as the actual test except that the homogenate was added just before precipitation after incubation.

The enzyme unit and its calculation

The splitting of steroid sulphates was calculated in μmoles of hydrolysed steroid sulphate per gram of tissue and hour and multiplied by 10^2, or in μmoles per milligram of nitrogen and per hour.

The number of units (U_W) per wet weight was obtained by multiplying percentage number of splitting with 100 k, in which $k = 0.0133$ for rat tissue that has hydrolysed DHAS, 0.533 for placenta that has hydrolysed DHAS and 2.67 for OS.

For nitrogen the number of units (U_N) was obtained in a corresponding way by multiplying with k the ratio of percentage number of splitting to mg of nitrogen in ml of 10 per cent homogenate.

C. Statistical calculations

The mean error of the difference of two independent determinations is calculated from formula

$$(1) \qquad s\{x' - x''\} = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (x_i' - x_i'')^2}$$

in which n is the number of pairs of determinations. The mean error of a single determination is thus

$$(2) \qquad s\{x\} = s\{x'\} = s\{x''\} = \frac{s\{x' - x''\}}{\sqrt{2}}.$$

For the calculation of the mean error

$$(3) \qquad s\{\bar{x}\} = \frac{s\{x\}}{\sqrt{n}}$$

of the mean

$$(4) \qquad \bar{x} = \frac{1}{n} \sum_{i=1}^{k} n_i x_i$$

the standard deviation $s\{x\}$ is calculated from the formula

$$(5) \qquad s^2\{x\} = \frac{1}{n-1} \sum_{i=1}^{k} n_i (x_i - \bar{x})^2,$$

in which x_i is the centre of class i, n_i its frequency, n the total frequency and k the number of classes.

The confidence limits of the difference between the mean of the populations of the quantities x and y $(\mu_x - \mu_y)$ at risk level p are

$$(6) \qquad \left(\bar{x} - \bar{y} - t_p \cdot s \sqrt{\frac{1}{n_x} + \frac{1}{n_y}},\ \bar{x} - \bar{y} + t_p \cdot s \sqrt{\frac{1}{n_x} + \frac{1}{n_y}}\right),$$

in which n_x and n_y are the total frequencies of the sampling of x and y,

$$(7) \qquad s^2 = \frac{(n_x - 1)\, s^2\{x\} + (n_y - 1)\, s^2\{y\}}{n_x + n_y - 2}$$

and t_p is obtained from the t-distribution table, the degrees of freedom being $n_x + n_y - 2$.

The regression line of y in regard to x is calculated from the formula

(8) $$y - \bar{y} = b_{yx}(x - \bar{x}),$$

where the regression coefficient of y on x

(9) $$b_{yx} = r \cdot \frac{s_y}{s_x},$$

in which s_x and s_y are the standard deviations of x and y and

(10) $$r = \frac{s_{xy}}{s_x s_y}$$

and in which, further

(11) $$s_{xy} = \frac{1}{n-1} \sum_{i=1}^{k} \sum_{j=1}^{l} n_{ij} (x_i - \bar{x}) (y_j - \bar{y}).$$

The confidence limits of the regression coefficient β_{yx} of the population at risk level p are

(12) $$b_{yx} \mp t_p \cdot \frac{s_y \sqrt{1 - r^2}}{s_x \sqrt{n-2}},$$

while the number of degrees of freedom is $n - 2$. They are always calculated at risk level $p = 0.05$ and entered in the above form.

To study the significance of the difference between the correlation coefficients, the following calculation was made

(13) $$z = \frac{1}{2} \ln \frac{1 + r}{1 - r},$$

the distribution of which is approximately normal with variance

(14) $$s^2\{z\} = \frac{1}{n-3}$$

and further

(15) $$\lambda = \frac{z_1 - z_2}{s\{z_1 - z_2\}}$$

in which

(16) $$s\{z_1 - z_2\} = \sqrt{s^2\{z_1\} + s^2\{z_2\}}$$

and the λ_p corresponding to risk level p is obtained from the normal distribution table.

The mean, the difference between two means, and difference of the regression or correlation coefficients is termined significant if $0.01 < p \leq 0.05$, highly significant if $0.001 < p \leq 0.01$ and very highly significant if $p \leq 0.001$.

EVALUATION OF THE METHOD

A. Arylsulphatase

Homogenate

The homogenate was made in distilled water because, as has already been mentioned, several anions and cations have activating and inhibiting effects. Since it was impossible to standardise the homogenisation completely, enzyme activity was studied in the same homogenate at the different phases of homogenisation. The results, given in Table IV, are the arithmetic means of three determination of the enzyme activity of 1 per cent rat homogenate.

Table IV. Effect of homogenisation degree on the enzyme activity.

Degree of homogenisation	μg of liberated p-nitrophenol	Range
very little	13.3	13.0—13.5
crude	17.7	15.2—21.7
normal	17.2	16.2—18.2
prolonged	15.7	15.0—16.0

It was possible also to make a rough microscopical classification into the four degrees of homogenisation used in the present work.

Activity was slightly lower in the very crude homogenates than in the others. Similarly, very prolonged homogenisation can result in inactivation, principally thermal.

The method of homogenisation employed in the present work can thus be regarded as suitable for the aims involved. The wet-weight percentage of the homogenate used is determined partly by the enzyme activity of the tissue, and in the present investigation especially by the small size of the tissues. When crude homogenates are used, 0.5—2 wet wt. per cent is the range most commonly employed in enzymology.

Inhibition often occurs in higher concentrations. In the reaction conditions of the present work, the relation between enzymatic activity and percentage of homogenate was fairly linear from 0.25 to 2 per cent

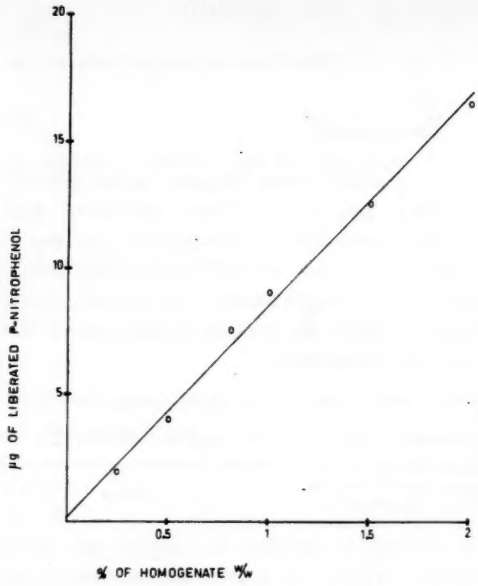


Fig. 2. The effect of the homogenate percentage used on the hydrolysis of the substrate. Rat liver in acetate buffer, pH 7.0; 0.001 M NPS.

(Fig. 2). If the homogenate percentage exceeds 3 no reliable results are obtained because of restrictions caused by the precipitation method and obvious protein inhibition.

Buffer

The effective range of acetate buffer ends around pH 6. Thus it was not an effective buffer for pH 7.3 and 7.0, the most commonly employed values in the present work. The literature contains some examples, however, of the use of acetate solution in arylsulphatase determination even at high pH values like these (Dodgson and Spencer 1957a, Dodgson, Spencer and Thomas 1953).

The present author checked the pH changes at the beginning and end of incubation. It might be expected that the products of hydrolysis originating in an enzymatic reaction are acid or basic, and that this would change the pH of the reaction milieu decisively during incubation. It appeared, however, that although p-nitrophenol was always liberated in amounts up to 30 µg in the reaction, the pH of the incubation solution did not change noticeably from 7.3. This shows that

no significant error arise in normal practice from the poor capacity of the buffer.

Optimum pH of the different tissues

The table compiled by DODGSON and SPENCER (1957b) shows a fairly great variety of pH optima for arylsulphatase. This is due to a great extent to the large number of substrates and enzyme sources used, the different buffers used and to the type of purified or unpurified fraction (fractions) measured.

The optimum pH of rat liver has been given as 6.12 (HUGGINS and SMITH 1947) and 6.6 (ABBOT and EAST 1949) in acetate buffer using crude homogenate and NPS substrate. The optimum pH of fraction C was 7.0 (DODGSON and THOMAS 1955) in 0.5 N acetate buffer with NPS substrate, that of fraction A 5.8 in similar conditions and of fraction B 6.2.

The literature gives no optimum pH values for crude homogenates of human tissue in acetate buffer with NPS as the substrate. The optimum pH of fraction C of human liver is 7.3 and of fraction A 6.2 (DODGSON and WYNN 1956).

The optimum pH of rat liver, kidney and intestine were studied in the reaction conditions of the present work. The value obtained for liver and intestine was c. 7.0, for the kidneys c. 5.8 (Fig. 3). The curves

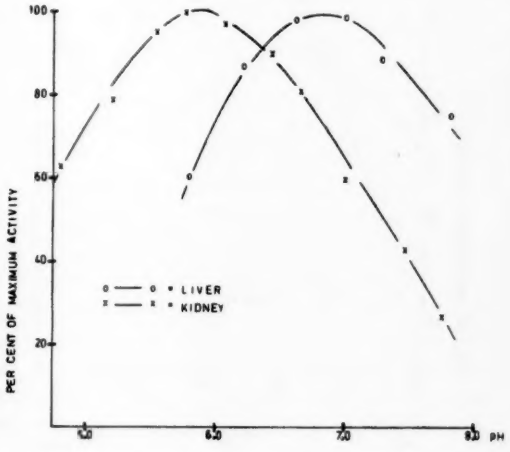


Fig. 3. Effect of pH on the activity of arylsulphatase in rat liver and kidney. The homogenate used for the liver was 1 and for the kidney 0.5 per cent wet wt. The substrate was 0.001 M NPS in acetate buffer.

in this work were plotted from the mean values of triple determinations and each triple determination was performed three times. The investigation of RUTENBURG and SELIGMAN (1956) is worth mentioning in this connection. These workers, using 6-benzoyl-2-naphthyl sulphate as substrate, obtained a more alkaline optimum pH than 6.4 (the highest pH they tried) for rat liver and the same value as above, i.e. 5.8, as the optimum pH for kidney.

The determination of the optimum pH in the acid milieu was complicated by the method of precipitation used in the present investigation. 0.8 ml of 0.5 N NaOH is incapable of alkalising an acetate buffer of under pH 6.2 sufficiently to produce the maximum colour in the liberated p-nitrophenol. In the actual test analyses, therefore, 0.63 N NaOH was used, and this was suitable. In the determination of the pH curves this source of error was taken into consideration by varying the normality of NaOH, but an excess of NaOH in these conditions can easily produce turbidity in the tubes after precipitation.

Optimum pH values were determined for the following human fetal tissues: liver, kidney, pancreas, intestines, lung and placenta. The optimum obtained for liver, pancreas, intestines and placenta was 7.3. Fig. 4 shows the results for liver and kidney. The optimum pH of

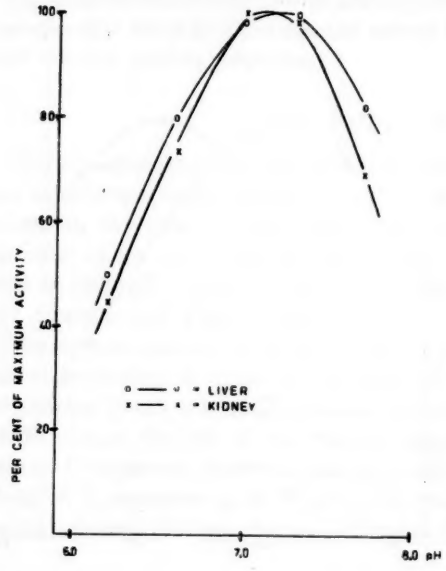


Fig. 4. pH activity curves for homogenaes of liver and kidney of human fetus, 0.005 M NPS substrate in acetate buffer.

kidney and lung is perhaps somewhat lower, 7.1, as can be seen from the curve obtained. These tissues were measured 6 times each at pH 7.3 and 7.1; pH 7.1 gave slightly higher results (c. 5 per cent). The result cannot be regarded as conclusive; it can only be said that the optimum pH is between 7.1 and 7.3. In practice, however, pH 7.1 was used for these tissues.

Substrate concentration

Substrate concentrations of 0.001 and 0.0005 M were used for rat liver in the literature in investigations using crude homogenates. In the investigation of HUGGINS and SMITH (1947) the optimal concentration obtained was 0.003 M. The optimal substrate concentration of fraction C of rat liver was 0.006 for NPS, and for fractions A and B 0.12 M. In the present work, 0.001 M was selected as the substrate concentration for practical reasons. Its blank value is also considerably smaller.

The optimum substrate concentration for NPS when crude homogenate of human tissue is used has not been determined earlier. For arylsulphatase fraction A of human liver the optimum is 0.07 M, of fraction C 0.008 M. Substrate concentration optima were determined in the present work for the liver and lung of the fetus. The same value was arrived at for both of them, 0.008 M, and this is the value given in the literature for fraction C. The results is shown in Fig. 5.

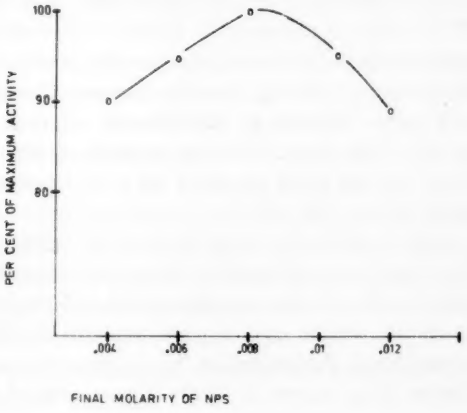


Fig. 5. The effect of the substrate concentration on the reaction velocity. Lung homogenate of human fetus (3 wet wt. per cent). NPS substrate, pH 7.1, acetate buffer.

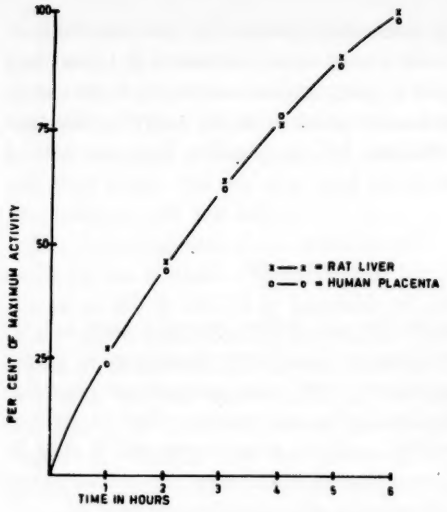


Fig. 6. The effect of incubation time on the reaction velocity. Homogenates of rat liver and human placenta (1 wet wt. per cent). For rat, 0.001 M NPS, pH 7.0; for man, 0.005 M NPS, pH 7.3. Acetate buffer.

Incubation temperature and time

The optimum incubation temperature for arylsulphatase has been given as 60° C (Abbot and East 1949). Rutenburg and Seligman (1956) observed that the reaction was most linear at 37° C. At 60° C the beginning of the reaction was rapid, but retardation occurred later on. The temperature generally used is in fact 37° C, and this was adopted for the present investigation.

The thermostat of the water bath kept the water temperature at 37° C with an accuracy of ± 0.5°. The samples had generally warmed up already at room temperature and were preincubated for 3—5 min. to achieve a constant reaction velocity throughout the test.

Enzyme activity as the function of time need not be linear over a very wide range. The curves obtained for rat liver show a relatively good but not exact linearity up to c. 3 hours, after which the reaction slows down (Fig. 6).

Fig. 6 shows the experiment with human placenta. The incubation time used was affected by the shape of the function-of-time curve and also by the relative enzyme content of the tissues. An endeavour was made to obtain an adequate amount of liberated p-nitrophenol for colorimetric determination by varying the incubation time. The incubation times shown in Table 3 were arrived at in this way.

Precipitation of the proteins

If protein precipitation is a two-phase procedure, as in the present work, the first chemical added must stop the reaction. If zinc sulphate is added first, 0.5 ml of 10 per cent solution, the enzymatic reaction is stopped almost completely, a c. 90 per cent inhibition occurs. Dodgson and Powell (1959) observed the inhibitory effect of zinc chloride also. However, it was found that the reaction did cease completely when sodium hydroxide was added. This practice was followed. It involves the possibility of NPS splitting non-enzymatically in the alkaline milieu (Dodgson and Spencer 1954). The serum of mammals contains a thermostable and non-dialysible factor which splits NPS non-enzymatically in a reaction. This is dependent on the strength of the alkalinity and on the temperature. If the amount of p-nitrophenol is determined immediately after the base has been and again 6 hours later, no notable differences will be found in these test conditions. However, the proteins were precipitated by adding zinc sulphate immediately, at least not later than 1 hour after the addition of sodium hydroxide.

Another risk involved in protein precipitation is that the liberated phenol, in this case p-nitrophenol, might be adsorbed into the precipitate. If $ZnSO_4$—NaOH precipitation is used by adding them in the ratio employed in this work, 100 per cent recovery of p-nitrophenol is obtained. This can be seen from the calibration curves obtained when water was substituted for the homogenate used in the experiment and when the homogenate was added in the usual way. The straight lines obtained converge.

The precipitation used in the present work is not effective if there is much protein. The highest homogenate percentage used (3 per cent) precipitates well without giving a disturbingly high blank value. The solution remains turbid if the percentage of the homogenate used exceeds 5.

When zinc sulphate was added to alkaline solution in the ratio used here, a precipitate originated which was first flocculent, then powdery. Filtering was performed immediately after adding $ZnSO_4$ because the powdery precipitate that had formed sometimes adsorbed p-nitrophenol and produced turbidity in the solutions to be measured.

The colour stability of the filtered green solution was good. Storage for 24 hours did not effect the intensity.

Metabolisation of the liberated p-nitrophenol and its maximum absorption

Caution is necessary in using NPS as the substrate for the fresh homogenate of rat liver since rat liver may metabolise p-nitrophenol liberated in an enzymatic reaction (DODGSON and SPENCER 1953a). On incubating rat liver homogenate and this phenol in acetate buffer these workers obtained only partial recovery. p-Nitrophenol solution thus incubated showed a small peak on the absorption curve at 290—295 mμ and the metabolisation product was identified by paper chromatography as p-aminophenol. Quantitative analysis showed that it made up for the deficit of the recovery test. The rat strain used by DODGSON and SPENCER was a "Medical Research Council Strain" which occasionally included albinos. It is to be noted that this phenomenon was not observed in rat intestine, nor was the mouse liver able to carry out this metabolisation. These phenomena are, however, fairly type- and strain-specific, as is shown by the arylsulphatase literature as a whole on many points concerning enzyme determination methods.

This phenomenon was checked occasionally for the rat strain used in the present work. 0.5 ml of 1 per cent rat liver homogenate in 1.5 ml of acetate buffer, pH 7.0, was incubated with 0.5 ml of p-nitrophenol aqueous solution, 15—30 μg of phenol per ml, for 3 hours, after which the proteins were precipitated in the usual way and the intensity of the colour measured at 402 mμ. In the control determinations the p-nitrophenol was added to the incubation mixture just before precipitation, keeping the other components constant; in other control experiments the homogenate was replaced by distilled water. Livers of rat fetuses, newborn rats and rats aged 1 month and adult rats of both sexes were used in the experiment. Three parallel determinations were made for the controls, too, and the experiment was repeated a couple of times in the course of the test series. No metabolisation of p-nitrophenol in the form of incomplete recovery was observed.

Furthermore, pure p-aminophenol, which is assumed to be a metabolite, was re-crystallised and decolorised by means of active carbon and the absorption curves of this p-aminophenol and incubated p-nitrophenol were compared. No peak was observed in the latter at the absorption maximum of p-aminophenol.

The ability of the liver or kidney of human fetus to metabolise p-nitrophenol was studied in the same way as for rat. No metabolisation was established in these cases either.

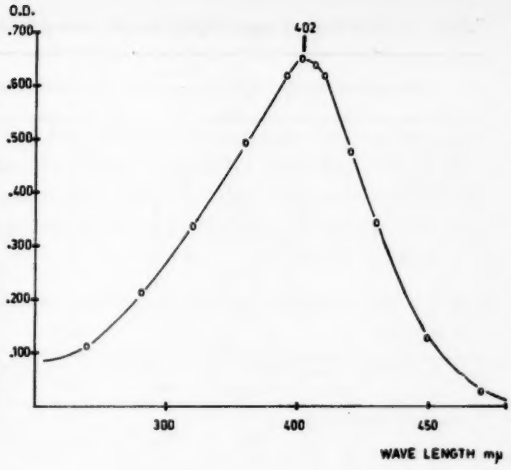


Fig. 7. The absorption curve of p-nitrophenol in alkaline solution. 1.5 ml of acetate buffer, pH 7.3, 0.5 ml of distilled water, 0.5 ml of aqueous solution of p-nitrophenol, 0.8 ml of 0.5 N NaOH and 0.5 ml of $ZnSO_4$; filtered.

The colour of p-nitrophenol has been measured at different wave lengths (Huggins and Smith 1947, Dodgson and Spencer 1953a). The absorption maximum varied slightly with the milieu. In the present work, the absorption maximum of p-nitrophenol was established as 402 mμ (Fig. 7).

Stability of the enzyme

The enzyme activity decreased to c. 75 per cent in an acetone suspension of marine molluscs when the solution was preincubated for 1 hour (Dodgson, Lewis and Spencer 1953). Activity did not decrease in a few hours at 0°C. It is generally held that arylsulphatase retains its stability long at —15°C (Dodgson, Spencer and Wynn 1956). Since there is no detailed information on this question which is an important one for the practical execution of the present work, the stability of the enzyme activity of human and rat homogenates was studied after keeping them in different concentrations for a day, a week and a month at room temperature (c. + 20°C), at + 4°C and at — 15°C. The results are shown in Table V. Arylsulphatase was less well preserved in a weak solution, which is a common finding. When kept at — 15°C no notable changes in activity occurred in 1 per cent homogenate. At + 20°C and + 4°C, on the other hand, the enzyme did not retain its activity for long and the later rise at room temperature has its natural explanation in the presence of this enzyme in many bacteria.

Table V. Stability of arylsulphatase in homogenate of human fetus and rat.

Duration of storage	Storage temperature	0.2 % human liver	1 % human liver	1 % human kidney	1 % rat liver
fresh	—	100	100	100	100
1 day	+20	2	25	62	32
„	+ 4	26	79	82	92
„	−15	55	110	90	93
1 week	+20	0	1	22	76*
„	+ 4	5	41	70	32
„	−15	35	104	85	72
1 month	+20	10*	2*	51*	—
„	+ 4	11	25	25	—
„	−15	38	81	78	60

* marked putrefaction.

Precision

It have been calculated the error of single determination for to study the arylsulphatase method.

Table VI. Error of a single determination of the method.

Ranges of μg liberated p-nitrophenol	0—2.5	2.5—5.0	5.0—10.0	10.0—20.0	over 20	all
n	57	57	55	56	56	281
$\bar{x}$	1.12	3.96	6.89	14.05	29.77	8.79
$s\{x\}$	0.226	0.53	0.60	1.16	1.83	0.90
%	20.2	13.3	8.8	8.3	6.1	10.2

n = number of determinations in each class
$\bar{x}$ = mean of the class
$s\{x\}$ = standard error of its single determination
% = $s\{x\}$ in per cent of $\bar{x}$

Table VI shows that the object in splitting NPS should be the highest percentage possible within the limits imposed by enzymological considerations proper. The mean accuracy of the parallel determinations in the present work was the same as obtained by KIRK and DYRBYE (1956) in a similar modification.

B. Hydrolysis of steroid sulphates

Regulation of the splitting

The technical procedure for making homogenate for hydrolysing steroid sulphates was the same as for arylsulphatase. The homogenate percentages and incubation times given in Table VII were selected in order to obtain suitable percentages in hydrolysis of steroid sulphate.

Table VII. Milieu used for the splitting of steroid sulphates. pH 7.8 and 0.2 mM steroid sulphate.

Type of tissue	Incubation time, hours			Homogenate percentage		
	DHAS	OS	AS	DHAS	OS	AS
rat liver	6	6	20	10	1	10
human placenta ..	3	3	20	5	1	10
tissues of human fetus	20	20	20	10	10	10

The aim was to achieve a splitting degree of c. 20 per cent. This would not change the substrate concentration to any appreciable extent but would permit accurate results. (cf. Table IX)

Purity

Unless the synthetic detergent used to wash the tubes is carefully rinsed out, a nonspecific addition of methylene blue or a complex of this dye and some foreign agent may take place in the chloroform phase and render the results entirely unreliable.

Reaction conditions

A substrate concentration of 0.2 mM has been used in the literature for both mammals and *Patella* enzyme. It was considered suitable also in the present work since there was nothing to suggest the contrary. There is no mention in the literature of the possible inhibition of TRIS buffer. The pH value used, 7.8, has been suggested as the optimum (Roy 1957). In the few determinations which were made at pH 6.0 and 8.5 using DHAS as the substrate, activity was observed to be smaller than at pH 7.8. Thus, by and large, this was the optimum also in the present investigation.

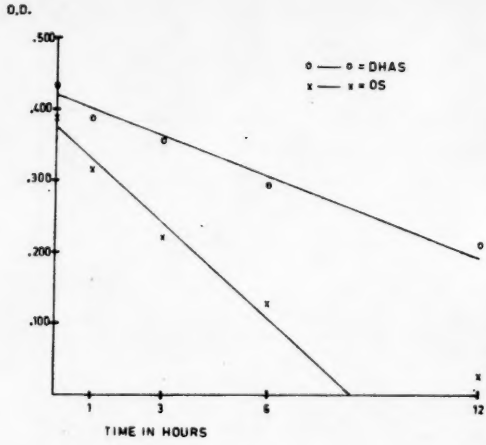


Fig. 8. Splitting of DHAS and OS by 10 and 1 wet wt. per cent rat liver homogenate, as a function of time. 0.2 mM steroid sulphate and pH 7.8 in TRIS buffer.

Reaction as a function of time

A 6-hour reaction time is generally to be considered long. For this reason, the splitting of DHAS and OS with rat liver homogenate was studied as the function of time. The results are shown in Fig. 8. For these conditions, the linearity can be considered relatively good. A decrease from the original substrate concentration down to 40 per cent does not seem to have any great effect on linearity.

Absorption spectrum of the complex and calibration

The maximum absorption of the steroid sulphate methylene blue complex has been given as 700 mμ (Roy 1957). Using the methylene blue of different manufacturers and different steroid sulphates, 663 mμ was obtained as the maximum absorption of the steroid sulphate methylene blue complex in alcohol solution (Fig. 9). Leon, Bullbrook and Corner (1960) later measured complexes at 655 mμ. The value obtained in this work as the absorption maximum for the complex in a non-alcohol solution was 658 mμ. The absorption maximum of aqueous solution of pure methylene blue measured against the solvent was 667 mμ.

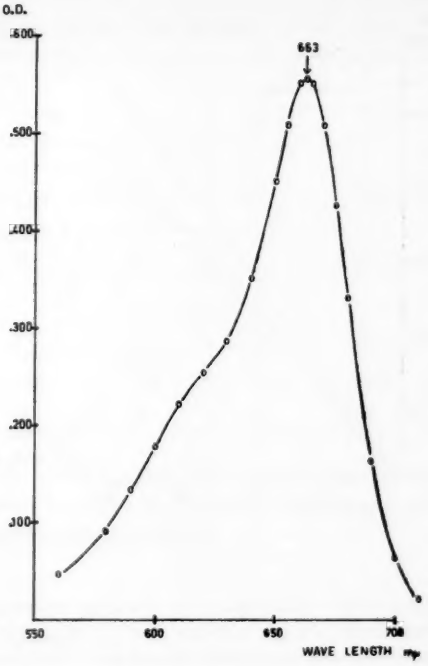


Fig. 9. Absorption curve of the complex formed by methylene blue and steroid sulphates, in chloroform-alcohol solution.

The formation of the complex by methylene blue and steroid sulphate and its extraction in chloroform were linear in the present study. The calibration curves obtained for DHAS, OS and AS are given in Fig. 10.

Stability of the hydrolysing ability of steroid sulphates

Rat liver homogenate was stored at a concentration of 10 per cent for DHAS and 1 per cent for OS. The times of storage of each sample, temperatures and their hydrolysing ability after a given period are shown in Table VIII.

The enzymes studied appear to be relatively stable, especially when kept in higher concentrations and at — 15°C; at room temperature, on the other hand, natural inactivation occurred.

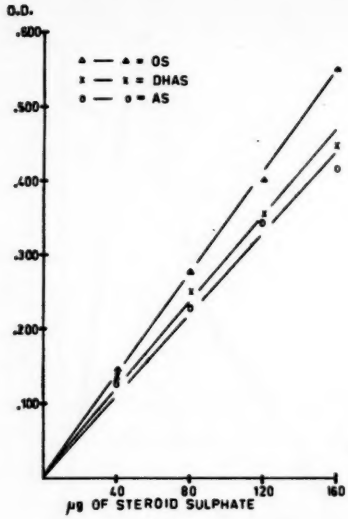


Fig. 10. Calibration curves for DHAS, OS and AS.

Table VIII. Stability of the splitting capacity of steroid sulphates. Rat liver. The results are given in per cent of steroid sulphate hydrolysed.

Duration of storage	Storage temperature	DHAS	OS
fresh	—	21.9	34.3
1 day	+20	16.9	26.5
„	+ 4	21.7	28.4
„	−15	21.7	25.6
1 week	+20	20.2	1.4
„	+ 4	21.7	34.7
„	−15	16.9	30.7
1 month	+20	−6.6	4.4
„	+ 4	4.5	16.2
„	−15	14.7	39.4

Precision

It have been calculated the error of single determination for to study the precision of steroid sulphate method.

Table IX. Error of a single determination of the method.

Ranges (% of split steroid sulphate)	0—10	10—20	20—30	over 30	Total
DHAS					
n	26	12	30	11	79
$\bar{x}$	5.4	14.7	24.2	33.5	17.9
s $\{x\}$	5.0	4.6	5.9	4.9	5.3
%	94	31	24	15	30
OS					
n	22	24	18	15	79
$\bar{x}$	5.4	15.0	25.8	39.7	3.9
s $\{x\}$	4.7	3.7	5.1	2.9	19.5
%	86.1	25	20	7	20

For the method to give fairly reliable results the splitting percentage should be at least 25. In order to achieve this the main factors varied were incubation time and hogomenate percentage.

RESULTS

A. Arylsulphatase

1. Human arylsulphatase

Localisation in different organs

The arithmetic means and the enzyme activities of the different organs of the fetus material as a whole are shown in Table X. The table also reveals the number of the determinations made per wet weight on the one hand and per nitrogen content on the other.

The enzyme was studied in human fetus in 26 different organs or secreta. The first observation made was that man shows relatively high arylsulphatase activity already in the fetal period. The localisation of the enzyme between the different organs was clear. It was most profuse in the liver, intestines, pancreas and kidney. In the few experiments to determine the arylsulphatase activity of the gallbladder it was found closest to that of the pancreas. The highest concentrations in the intest-

Table X. The amount and localisation of arylsulphatase in human fetal organs. Crown-heel length of fetus 0.5—33 cm.

Tissue	Calculation per wet weight (U_w)		Calculation per tissue nitrogen content (U_N)	
	$\bar{x} \pm s \{\bar{x}\}$ (n)	range	$\bar{x} \pm s \{\bar{x}\}$ (n)	range
liver	1260±71 (64)	540—3220	770±50 (55)	270—2300
ileum	675±50 (40)	186—1620	410±36 (39)	80—1470
jejenum	600±45 (41)	222—1380	390±31 (37)	160—820
colon	420±44 (41)	103—1400	194±12 (33)	65—520
duodenum	373±29 (43)	130—1080	310±32 (37)	75—750
intestines (smallest)	232±28 (19)	66—480	240±54 (8)	65—350
pancreas	186±17 (33)	60—400	134±12 (27)	55—240
kidney	124±8.9 (71)	3—300	118±8.1 (51)	16—300
lungs	40.3±3.6 (59)	3—133	47±3.7 (48)	5—96
stomach	37.6±2.6 (45)	6—102	29±3.1 (29)	6—93
brains	13.5±1.24 (54)	3—36	17.7±1.48 (48)	1—41
genitals	11.5±1.65 (29)	1—32	10.0±1.76 (22)	2—35
salivary gland	9.9±2.51 (26)	1—66	10.3±1.68 (21)	1—37
adrenal gland	8.9±0.89 (59)	1—24	6.5±0.57 (42)	1—16
heart	7.1±0.94 (48)	1—31	5.0±0.70 (39)	1—21
thyroid gland	6.0±0.92 (35)	0—13	5.1±0.71 (34)	0—16
skeletal muscle	5.8±0.71 (27)	0—12	5.3±0.67 (21)	0—10
thymus	4.8±0.65 (35)	0—14	3.6±0.54 (29)	0—12
spleen	4.4±0.41 (41)	0—13	2.3±0.44 (28)	0—11

ines were around the middle of it, in the jejunum and in the ileum. The concentration was higher in the latter, but the difference between the two parts was not statistically significant. The colon and the duodenum were nearly equally rich in the enzyme. There was relatively little activity in the upper part of the digestive tract, the stomach. Pulmonary tissue contained less enzyme than the poorest of the tissues above, the kidney, but its activity was greater than that observed in the brains, especially in larger fetuses. No significant differences were observed in the few cases in which the different parts of the brains were analysed. The genitals revealed a distinctly demonstrable enzyme content in the fetal period. Activity figures of the same magnitude were obtained from the oviduct, the testis, the uterus and the genitals in general. Weak activity was established in the cardiac muscle, and the skeletal muscle

had hardly any enzyme. With the exception of the pancreas the glandular tissues do not possess much arylsulphatase. The highest activities were established in the sexual glands of fetuses and the salivary glands (gl. parotis) of larger fetuses. The adrenal gland also displayed weak activity. It was impossible to establish any definite enzyme activity towards NPS in the thyroid gland, the spleen and the thymus. The values recorded for them approximated to a technical potential error. Nor was any activity established either in the bone marrow or in the bones themselves. No activity was present in the walls of the three urinary bladders examined.

Of the secreta, meconium had a fairly large amount of arylsulphatase. No activity was established in the urine or amniotic fluid. The method is not suitable for examination of the blood because of its high protein content.

Arylsulphatase in the organs during the organogenetic period of human fetus

Liver

The arylsulphatase activity of 64 fetus livers was studied and the nitrogen content of the liver homogenate determined in 55 of them. The crown-heel length of the fetuses ranged from 2.0 to 33.0 cm, mean 11.8 cm. The mean enzyme activity established was 1260 ± 70 U_w and 770 ± 50 U_N. The ranges were 540—3220 U_w and 270—3200 U_N. The regression between enzyme activity and length is shown in Fig. 11 where the results were calculated per wet weight. No statistically significant regression was observed between length and activity with either method of calculation.

Alimentary canal

(a) *Alimentary canal as a whole.* It was considered impossible in 19 fetuses under 9 cm in length to distinguish with sufficient accuracy between the different parts of the alimentary canal, and enzyme activity was consequently determined from the whole. Because of the small size of these fetuses, tissue nitrogen was determined in 8 cases only. The

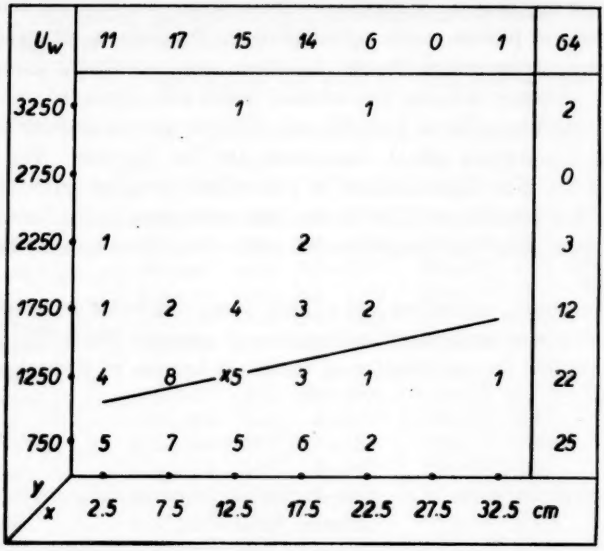


Fig. 11. The regression of the arylsulphatase activity of human liver to fetal length, calculation per wet weight. $p > 0.05$.

length of the fetuses ranged from 2.5 to 7.9 cm, mean 5.5 cm. The mean enzyme activity of the alimentary canal as a whole was 232±28 U_w and 240±54 U_N. The regression as regards fetus size was statistically significant per wet weight. There was no regression as regards nitrogen, but the series was rather small in this group, only 8 fetuses.

(b) *Stomach.* The material consisted of 45 stomach walls in 29 of which tissue nitrogen was determined. The fetus length ranged from 8.5 to 33 cm, mean 13.9 cm. The smallest arylsulphatase activity in the digestive tract was that of the stomach, an average of 37.6±2.6 U_w and 29±3.1 U_N. The ranges were 6—102U_w and 6—96 U_N. The regression between growth and arylsulphatase content was statistically highly significant in the stomach wall calculated per wet weight ($r = 0.459 \pm 0.118$, $p = 0.01$); activity rose 1.2 ∓ 0.64 U_w/cm. In terms of tissue nitrogen, however, it was impossible to establish the same correlation and the rise was thus ostensible.

(c) *Duodenum.* The duodenum of 43 fetuses was studied and in 37

cases a tissue nitrogen determination was performed. The fetus length was 6.5—28.5 cm, mean 14.9 cm. The enzyme activity established was considerably greater than the comparable activity in the stomach wall, 373 U_w and 310 U_N, while the standard deviations of their means were 29 U_w and 32 U_N. The ranges were 130—1080 U_w and 75—750 U_N. There was no regression as regards length.

(d) *Jejunum.* Forty one jejunums were examined and 37 type analyses made of them. The length of the fetuses was 7.5—28.5 cm, mean 15.2 cm. The arylsulphatase activity was 600 ± 45 U_w and 390 ± 31 U_N. The regression between enzyme activity and the fetal length was not statistically significant.

(e) *Ileum.* The arylsulphatase activity of 40 fetus ileums was determined, and for 39 of them the tissue nitrogen was determined. The length of the fetuses was also 7.5—28.5 cm, mean 15.6 cm. The enzyme content was 675 ± 50 U_w and 410 ± 36 U_N. The activity of the ileum was thus somewhat greater than the jejunum activity, but the difference was not statistically significant. The regression between enzyme activity and fetal length was not statistically significant.

(f) *Colon.* The material consisted of 41 fetus colons and 33 of them were analysed for tissue nitrogen. The fetuses were the same as those used in determining jejunal enzyme activity. The contents were 420 ± 44 U_w and 194 ± 12 U_N. The arylsulphatase activity of the colon was statistically very highly significantly lower than in the jejunum and ileum. No regression in relation to fetal length was established in this part of the alimentary canal either.

Pancreas

The pancreas of 33 fetuses was examined. Tissue nitrogen was determined in 27 of them. The crown-heel length of the fetuses was 8.5—33.0 cm, mean 16.4 cm. The average enzyme activity in the pancreas was 186 ± 17 U_w and 134 ± 12 U_N, limit values 60—400 U_w and 55—240 U_N. No significant increase was established in enzyme activity with the growth of the fetus.

Kidney

The kidney of 71 fetuses was analysed and the tissue nitrogen determined in 51 of them. The fetus length was 2.0—33.0 cm, mean 12.4 cm. Enzyme activities of the following magnitude were established in the

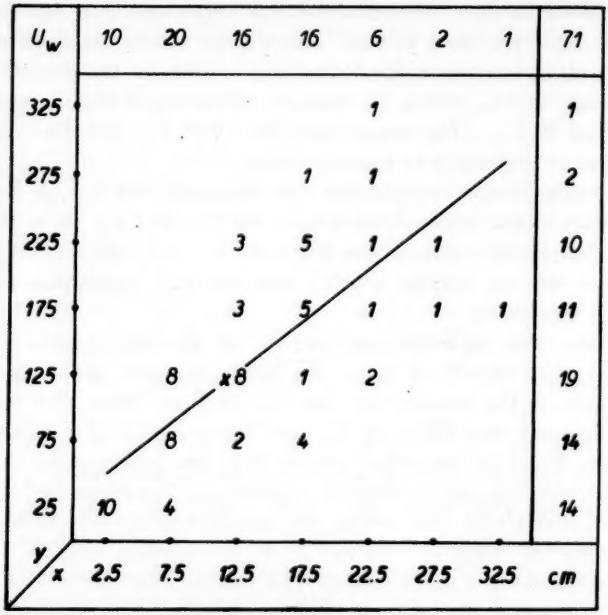


Fig. 12. Regression of the arylsulphatase activity of human kidney to fetal length; calculation per wet weight. $p < 0.001$.

kidney: 124 ± 8.9 U_w and 118 ± 8.1 U_N. The limit values were 3—300 U_w and 16—300 U_N. Enzyme activity increased statistically very highly significantly with fetal growth, per wet weight and per tissue nitrogen ($r = 0.713 \pm 0.058$, $p < 0.001$ calculated per wet weight and $r = 0.716 \pm 0.068$, $p < 0.001$ calculated per tissue nitrogen). The regression between activity and length is shown in Fig 12. The regression line gives the increase as 7.67 ∓ 0.908 U_w/cm and 6.44 ∓ 0.755 U_N/cm.

Lung

The material included 59 fetuses and 48 nitrogen analyses were made of the lung homogenate. The crown-heel length of the fetuses was 2.5—28.5 cm, mean 11.4 cm. The observed arylsulphatase activity varied from 3 to 130 U_w and 5—96 U_N, means 40.3 ± 3.6 U_w and 47 ± 3.7 U_N. The arylsulphatase content increased both per wet weight

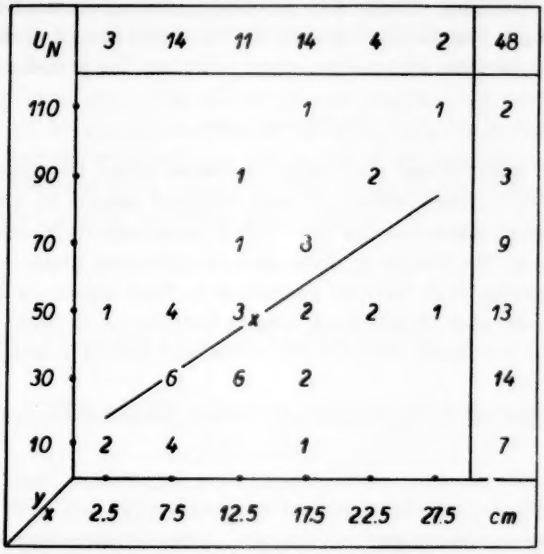


Fig. 13. The regression of the arylsulphatase activity of human lung to fetal length; calculation per nitrogen content. $p < 0.001$.

and per tissue nitrogen content as the fetus developed. The regression between activity and length was statistically very highly significant by both methods of calculation ($r = 0.605 \pm 0.082$ and $p < 0.001$ and $r = 0.618 \pm 0.089$ and $p < 0.001$, respectively). The values of the regression line per wet weight were $b_{yx} = 2.7 \mp 0.936$ U_w/cm and per tissue nitrogen 2.59 ∓ 0.977 U_N/cm. The regression line for pulmonary enzyme activity and fetus length is given in Fig. 13. The correlation coefficient for the enzyme activity of the lung and fetal length did not differ statistically significantly from that for the kidney; in other words, enzyme activity increased linearly as good in the lung and in the kidney during the period of fetal organogenesis studied.

Brain

The brain showed some arylsulphatase activity, though it was fairly small. The material consisted of 54 fetuses for 48 of which the brain tissue nitrogen was determined. Activity varied from 2.5 to 30.8 U_w

and 6.5 — 41.0 U_N, means 13.5 ± 1.24 U_w and 17.7 ± 1.48 U_N. The regression as regards the length of the fetus was not significant. No notable increase in activity occurred during the period studied.

Other tissues

Enzyme activity was very small in tissues other than these tissues detailed above. Such values as were obtained cannot be regarded as fully reliable quantitatively since they were relatively close to the blank values. The results in these cases are therefore given in the form of 3 categories only, divided according to fetal length: under 9 cm, 9—16 cm and over 16 cm. These lengths were chosen as they correspond roughly to the limits between the third and fourth months of pregnancy.

The results obtained are given in Tables XI and XII.

Table XI. The enzyme concentrations of some arylsulphatase-poor tissues, by fetal size groups; calculation per wet weight $\bar{x} \pm s \{\bar{x}\}$ (n)

Tissue	Under 9 cm	9—16 cm	Over 16 cm
genitals	10.8 (2)	9.4±1.65 (14)	14.1±2.8 (11)
salivary gland ...	2.2 (3)	7.7±1.16 (10)	14.3±5.6 (10)
adrenal gland ...	8.5±0.96 (18)	10.2±1.67 (23)	7.7±1.8 (15)
heart	7.5±1.52 (18)	8.7±1.89 (15)	4.6±1.14 (12)
thyroid gland ...	3.9±0.86 (4)	5.5±1.04 (16)	7.4±1.9 (12)
skeletal muscle ..	6.3 (2)	5.0±0.85 (16)	5.7±1.5 (7)
thymus	—	4.6±0.30 (20)	2.2±0.81 (12)
spleen	2.3±0.49 (8)	5.5±0.59 (17)	4.3±0.69 (14)

Table XII. The enzyme concentrations of some arylsulphatase-poor tissues by fetal size groups; calculation per tissue nitrogen content. $\bar{x} \pm s \{\bar{x}\}$ (n).

Organ	Under 9 cm	9—16 cm	Over 16 cm
genitals	3.5 (1)	10.4±3.1 (10)	10.3±2.0 (11)
salivary gland ..	—	9.5±1.55 (11)	11.2±1.03 (10)
adrenal gland ..	8.1±1.3 (9)	6.3±0.77 (19)	5.7±1.03 (14)
heart	4.8±1.43 (14)	5.8±1.05 (15)	4.2±1.20 (10)
thyroid gland ...	2.3 (1)	9.5±0.93 (21)	4.6±1.19 (12)
skeletal muscle ..	6.3 (2)	5.0±0.87 (12)	5.7±1.34 (7)
thymus	—	4.6±0.78 (17)	2.2±0.57 (12)
spleen	—	2.8±0.73 (16)	1.4±0.34 (12)

Arylsulphatase was generally found during the fetal period in the genitals. Activity was greater on the whole in large (over 16 cm) fetuses than in the other two categories but the difference was not statistically significant. The salivary glands possessed the same amount of activity as the genitals. In the thyroid gland and the cardiac muscle there was perhaps some activity, but for the spleen and skeletal muscle the values obtained were below the lower limit of the method and no actual enzyme activity could be said to exist.

2. Arylsulphatase in human placenta

Arylsulphatase activity of the placenta during the earlier fetal period

(a) Fetal side. The enzyme activity on the fetal side was determined in 55 placentas. The placentas belonged to fetuses with a crown-heel length of 0.5—33 cm, mean 12.1 cm. The nitrogen content of the homogenate was analysed for 40 of them. The mean arylsulphatase content was 191 ± 11.2 U_w and 183 ± 14.1 U_N. The wet weight values gave a statistically significant regression in which $b_{yx} = 3.86 \mp 3.42$ U_w/cm. The correlation coefficient was 0.298, $p = 0.05$. The tissue nitrogen values, however, gave a regression that was not statistically significant.

(b) Uterine side. Samples were taken from the placenta of 50 fetuses with a crown-heel length of 0.5—33 cm, mean 11.9 cm. The nitrogen content of 32 samples was determined. The activity observed was 171 ± 12.1 U_w and 156 ± 15.6 U_N. The activity of arylsulphatase grew in the placenta also on the uterine side as the fetus grew (4.1 ∓ 3.57 U_w/cm, $r = 0.318$, $p = 0.05$). The result was statistically significant.

It was impossible here again to observe a significant increase in activity from the results calculated per nitrogen content.

There was a statistically highly significant difference between the activity on the fetal and on the uterine side of the placenta in the results calculated per wet weight; per tissue nitrogen there was only a tendency to greater activity on the fetal than on the maternal side.

Arylsulphatase of the placenta at the time of delivery

The samples taken from the maternal side showed a mean activity of 241 ± 15.1 U_w and 104 ± 5.2 U_N, those from the fetal side of 218 ± 17 U_w and 86.6 ± 6.90 U_N. Taking each placenta separately, the difference between these two means was significant both per wet weight and per tissue nitrogen content ($p = 0.05$). There was more arylsulphatase on the mother's side. The wet weight value for activity was greater in the placenta at the time of delivery than in the earlier fetal period. The difference was statistically highly significant ($p < 0.01$). This was not the case with the values calculated per tissue nitrogen content; the situation was the reverse, the difference highly significant.

The series included fraternal twins whose placental activity was close to the mean for the series and near each other (256 and 280 U_w and 258 and 288 U_N). It also contained 2 placentas of subjects with mild pre-eclampsia in which the activity did not differ notably from the general level.

3. Rat arylsulphatase during intra- and extrauterine development

Intrauterine development

(a) Liver. The enzyme content of the liver of 65 fetuses was analysed per wet weight. The weight of the fetuses was 0.5—5.56 g, mean 2.4 g. The mean arylsulphatase activity in the liver of rat fetus was 16.0 ± 1.12 U_w. There was a statistically very highly significant regression between the total weight of the fetus and activity, i.e. 2.80 ∓ 1.14 U_w/g in which $r = 0.525$ and $p < 0.001$. The regression curve is shown in Fig. 14.

It was possible determine the tissue nitrogen content in the livers of 43 fetuses. The total weight range in this group was 0.77—5.5 g, mean weight 2.97 g. The fetuses were a little larger as the homogenate from the smaller fetuses did not suffice for the nitrogen determination. The mean activity was 9.7 ± 0.27 U_N. The regression was statistically highly significant, even when calculated in this way, an increase of 1.48 ∓ 1.08 U_N per gram ($r = 0.390$, $p < 0.01$). The increase in the

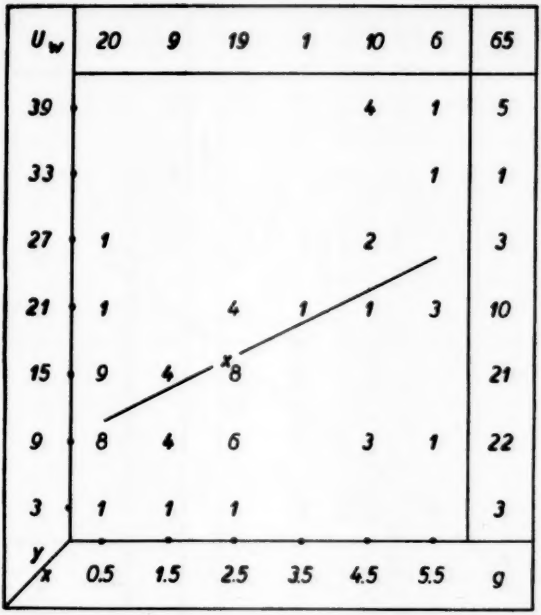


Fig. 14. The regression of the arylsulphatase concentration of rat liver to fetal weight; calculation per wet weight. $p < 0.001$.

tissue nitrogen value was not as sharp as in the wet weight values. *(b) Kidney.* Activity determinations per wet weight were performed for the kidneys of 49 fetuses of different sizes. The fetal weight range was 0.77—5.51 g, mean 2.84 g. Owing to the small size of the kidneys of fetuses under 1.5 g, the kidneys of several fetuses of the same mother were pooled for analysis and the mean fetal weight was entered as the size. The arylsulphatase activity of rat kidney in the fetal period was thus assessed as 12.7 ± 0.97 U_w. Growth in fetal size was accompanied by very highly significant increase in enzyme content, 2.96 ∓ 1.01 U_w/g ($r = 0.619$, $p < 0.001$). The results are shown in Fig. 15.

The tissue nitrogen content was determined in 28 fetuses weighing 1.98—5.56 g, mean 2.97 g, i.e. only the largest individuals. The mean activity was 7.62 ± 1.11 U_N. These large fetuses revealed no statisti-

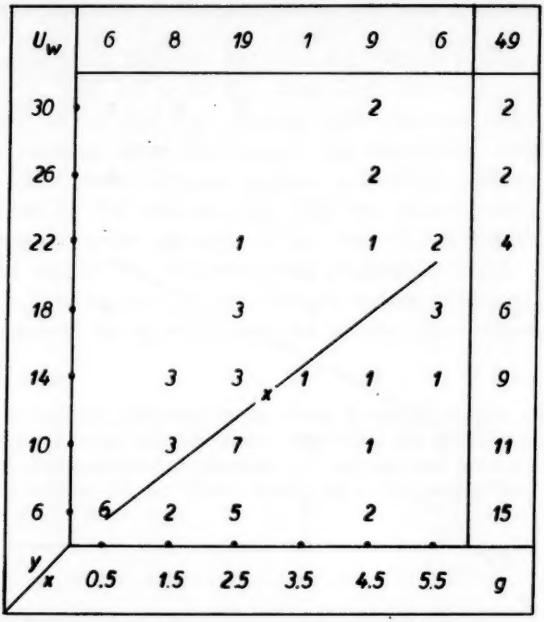


Fig. 15. The regression of the arylsulphatase concentration of rat kidney to fetal weight; calculation per wet weight. $p < 0.001$.

cally significant regression as regards size, although the same tendency was observable.

(c) Alimentary canal. The enzyme activity of the alimentary canal of g 0.61—5.56 weighing fetuses was measured. The mean weight was 2.26 g, enzyme activity 6.3 ± 0.31. The regression was 0.77 ∓ 0.32 U_w/g ($r = 0.522$, $p = 0.01$). The growth of activity with the increase in weight was thus statistically highly significant.

The alimentary canals of 43 rat fetuses were analysed for tissue nitrogen. The fetal weight range was 0.77—5.56 g, mean 2.82 g. The mean enzyme content was 4.6 ± 0.25 U_N/g, but the regression in regard to size was not statistically significant.

Table XIII. The arylsulphatase activity of rat liver, kidney and intestine by age groups.
$\bar{x} \pm s \{\bar{x}\}$ (n)

Age	Liver		Kidney		Intestine	
	(U_w)	(U_N)	(U_w)	(U_N)	(U_w)	(U_N)
fetus under 4 g	12.9±0.86 (49)	9.8±0.95 (43)	12.7±0.97 (49)	5.6±0.68 (16)	5.8±0.30 (46)	3.72±0.34 (39)
fetus over 4 g	24.6±3.3 (15)				7.5±0.67 (16)	
under 24 hours	26.1±1.75 (46)	11.9±0.87 (46)	32.5±2.2 (28)	17.7±1.93 (22)	8.1±0.60 (44)	3.9±0.32 (42)
1 month	70.3±3.8 (36)	24.9±1.58 (38)	73.1±8.1 (48)	22.0±0.67 (29)	7.2±0.71 (47)	2.4±0.22 (33)
3 ½ month	109±4.4 (54)	38.3±1.83 (43)	81.0±2.56 (55)	28.8±1.41 (42)	6.5±0.65 (52)	3.3±0.56 (40)

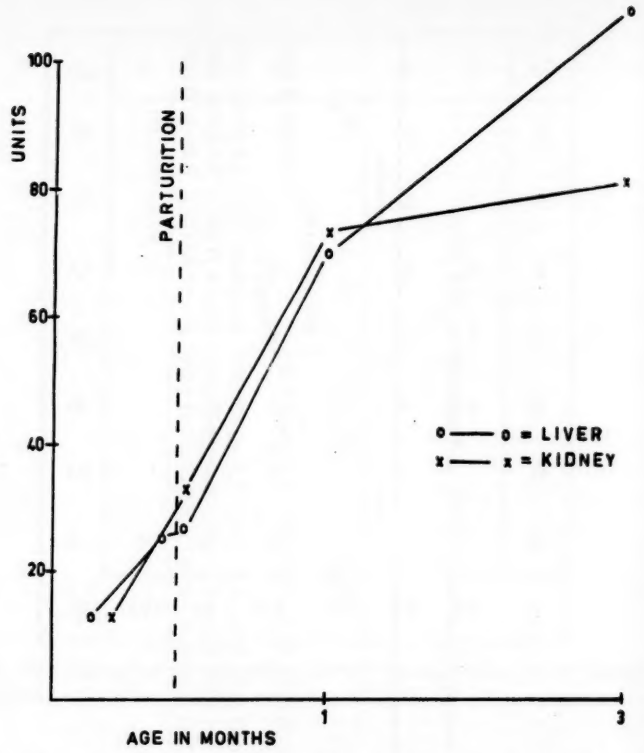


Fig. 16. Rat arylsulphatase as a function of age, in liver and kidney. Substrate 0.001 M NPS, acetate buffer, pH 7.0 for liver and 5.8 for kidney, and homogenate percentages 1.0 and 0,5, respectively. μg of liberated p-nitrophenol/100 mg wet wt./hr.

Enzyme during extrauterine development

Arylsulphatase concentrations in different age groups

Table XIII summarises the activities by age groups. The results for the liver and the kidney are presented graphically in Fig. 16.

(a) Newborn. The liver of 46 newborn rats was studied and analysed for tissue nitrogen content. Enzyme activity was 26.1 ± 1.7 U_w and 11.9 ± 0.87 U_N. The kidney contained 32.5 ± 2.2 U_w and 17.7 ± 1.9 U_N

and the intestine 8.18 ± 0.60 U_w and 3.9 ± 0.32 U_N. Comparing the results with fetus enzyme concentrations in general, the difference in the liver per wet weight was very highly significant, activity being higher in the newborn. Making a similar comparison for the nitrogen unit values, the increase was not statistically significant ($t = 1.75$), but the same tendency could be observed. Because of the small size of the fetuses, tissue nitrogen determinations were possible only for the major fetuses (mean weight 2.97 ± 0.27 g against 2.40 ± 0.21 g) and besides this the activity rose very highly significantly per tissue nitrogen as the fetus grew.

In order to demonstrate possible changes in enzyme content at the moment of birth, the liver arylsulphatase activity of fetuses weighing over 4 g was calculated. It was 24.6 ± 3.3 U_w (15 analyses). The concentration was fairly close to that established for newborn rats (26.1 ± 1.7 U_w); the difference was not significant.

The kidney arylsulphatase content of newborn rat was very highly significantly greater than that in fetuses, both per wet weight and per tissue nitrogen content.

In the intestines the concentrations were consistently very low, at the lower limit of the method. No significant difference from the fetal values was established as regards tissue nitrogen. As regards wet weight values, fetuses under 4 g in weight showed a very highly significantly smaller amount of arylsulphatase; the difference from the larger fetuses was not significant.

(b) Rats aged 1 month. The arylsulphatase concentrations established in 48 rats were 70.3 ± 3.8 U_w and 24.9 ± 1.58 U_N in the liver, 73.1 ± 8.1 U_w and 22.0 ± 0.69 U_N in the kidney and 7.2 ± 0.71 U_w and 2.4 ± 0.22 U_N in the intestine. Compared with newborn rats, the increase in activity was statistically very highly significant in the liver by both methods of calculation. The same was true of the kidney in regard to wet weight, but in terms of tissue nitrogen the difference was only significant. A significant decrease occurred in the intestinal concentrations in regard to tissue nitrogen; as regards wet weight, the difference was not significant.

(c) Adult rats aged 3 ½ months. The enzyme activity in 54 rats of this group was 109 ± 4.4 U_w and 38.3 ± 1.83 U_N in the liver, 81.0 ± 2.56 U_w and 28.8 ± 1.41 U_N in the kidney and 6.5 ± 0.65 U_w and 3.3 ± 0.56 U_N in the intestine. The increase in the liver was statistically very highly significant both per wet weight and per tissue

Table XIV. Rat arylsulphatase concentrations by sexes. $\bar{x} \pm s$ $\{\bar{x}\}$ (n)

	(U_w)	(U_N)
liver of rat aged 1 month,		
female	66±5.3 (16)	22.9±2.6 (14)
male	74±5.4 (20)	26.1±2.5 (18)
liver of rat aged 3 ½ months,		
female	99±6.3 (26)	38.7±3.0 (19)
male	116±5.4 (30)	38.1±2.6 (23)
kidney of rat aged 1 month,		
female	58±5.3 (16)	21.5±1.2 (15)
male	58±4.6 (19)	20.8±1.35 (14)
kidney of rat aged 3 ½ months,		
female	87±3.0 (28)	32.2±2.3 (19)
male	75±4.0 (27)	27.4±1.61 (23)

nitrogen compared with rats aged 1 month. The increase was significant in the kidney per wet weight and very highly significant per tissue nitrogen. There were no significant changes in the intestinal concentrations.

Sex difference

The mean enzyme concentrations in rats of either sex aged 1 and 3 ½ months are given in Table XIV for the liver and kidneys.

The sex difference was not statistically significant in either organ in this strain of rat at the age of 1 month. Adult rats, on the other hand, according to the wet weight values, showed a statistically significantly greater amount of enzyme in the liver and a significantly smaller amount in the kidney of male. No such difference could be demonstrated in the results per tissue nitrogen.

B. Hydrolysis of steroid sulphates

Hydrolysis in growing rat organism

The means and standard errors for the different age groups are summarised in Table XV. It shows the splitting of both DHAS and OS measured in terms of wet weight and tissue nitrogen, and the number of analyses made. The changes in enzyme activity are also presented graphigally in Fig. 17.

Table XV. Splitting of DHAS and OS by rats of different ages.

	DHAS		OS	
	(U_w)	(U_N)	(U_w)	(U_N)
fetus	1.1±1.23 (23)	—	8±25 (23)	—
newborn ..	1.4±1.24 (17)	—	230±47 (16)	89±16 (16)
1 month ..	29.3±1.62 (28)	10.8±1.10 (28)	440±40 (27)	155±16 (27)
3½ months	36.4±1.33 (30)	13.3±1.00 (30)	990±55 (28)	360±21 (28)

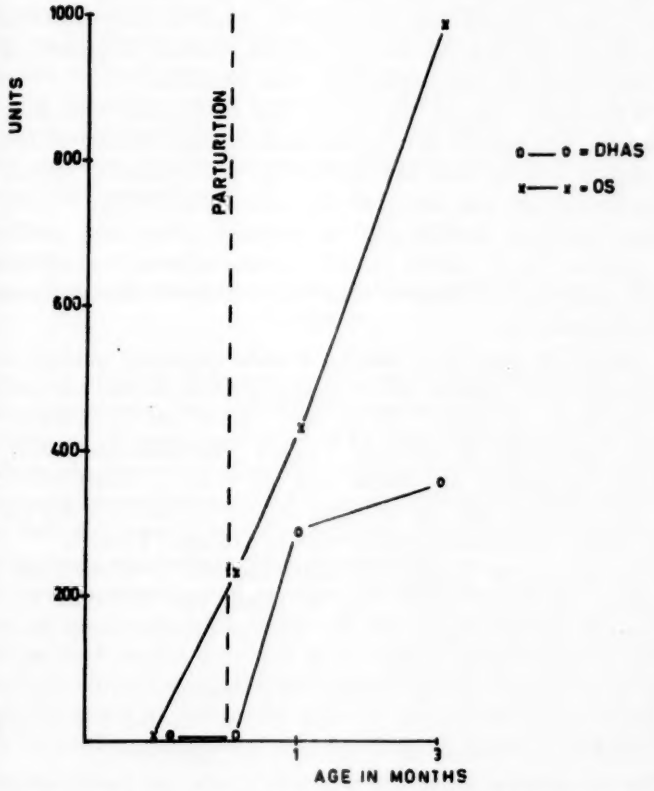


Fig. 17. Splitting of DHAS and OS in the liver of growing rat. 0.2 mM steroid sulphate, pH 7.8 in TRIS buffer. Results for DHAS multiplied with 10.

(a) *Fetuses.* The livers of 29 fetuses of different ages were analysed for DHAS, giving 1.1 ± 1.23 U_w as the mean steroid sulphatase activity. This was not significantly different from 0 and the values for activity were both positive and negative. Nor was the mean (8 ± 25) for OS significantly different from negative; the results were both under and above 0. Steroid sulphates were not hydrolysed by the liver of rat fetus in these experimental conditions.

(b) *Newborn.* The DHAS splitting ability of the liver of 16 newborn rats was 1.4 ± 1.24 U_w. This was not significantly different from 0: both positive and negative values were obtained.

Only positive values were obtained for the hydrolysis of OS. The mean enzyme activity was 230 ± 47 U_w and 89 ± 16 U_N. The rat of newborn age was capable of splitting estrone sulphate but not dehydroepiandrosterone sulphate in these conditions.

(c) *Rats aged 1 month.* Of the 28 rats studied, 14 were male and 14 female. The specific steroid sulphatase content in rats of this age was 29.3 ± 1.62 U_w and 10.8 ± 1.10 U_N. The amount of estrone sulphate hydrolysed was 440 ± 40 U_w and 155 ± 16 U_N. Steroid sulphatase was thus demonstrable; no negative values were established The increase in the ability to split estrone sulphate was statistically highly significant in terms of both wet weight and nitrogen compared with newborn rats.

(d) *Adult rats aged 3 ½ months.* Steroid sulphatase analysis of the livers of 30 rats, 15 male and 15 female, gave an enzyme concentration for the group of 36.4 ± 1.33 U_w and 13.3 ± 1.00 U_N. Activity was a little higher than in rats aged 1 month. The difference was not statistically significant. The means were 990 ± 55 U_w and 359 ± 21 U_N for OS. In adult rats the splitting activity of the liver for OS was very highly significantly greater than in rats aged 1 month.

(e) *Sex difference.* The steroid sulphatase activity of male rats aged 1 month was 31.4 ± 4.5 U_w and of female rats 27.8 ± 4.5 U_N. The difference was not significant. Nor was the difference significant per tissue nitrogen content (10.8 ± 1.52 and 10.8 ± 1.61 U_N). For adult male and female rats the corresponding enzyme activity means were 36.3 ± 4.13 and 37.3 ± 3.81 U_w and 13.1 ± 1.55 and 13.5 ± 1.23 U_N. The differences between the sexes were not significant.

The OS splitting activity in male and female rats aged 1 month was 440 ± 56 and 390 ± 64 U_w and 162 ± 26 and 150 ± 20 U_N. The differences were not statistically significant. The corresponding values for

adult rats were 950 ± 24 and 1020 ± 53 U_w, and 360 ± 29 and 355 ± 33 U_N. The differences were not significant. It was thus impossible with the methods and rat strain used to establish sex differences in the hydrolysis of steroid sulphates.

Human placenta as a splitter of steroid sulphates

Specific steroid sulphatase was found to be very profuse in the human placenta. The mean amount was 173 ± 20 U_w and 75 ± 7.4 U_N on the maternal side and 187 ± 20 U_w and 71 ± 4.5 U_N on the fetal side. The amount was larger than e.g. the rat liver concentrations ($p < 0.01$). There was no significant difference in localisation between the two sides.

Estrone sulphate was also split vigorously by placenta homogenates. The unit quantities obtained were 860 ± 71 U_w and 345 ± 32 U_N on the maternal side and 885 ± 80 U_w and 350 ± 34 U_N on the fetal side. The means were roughly the same on both sides and the difference was not statistically significant.

Human fetus and steroid conjugates, and the hydrolysis of androsterone sulphate

The liver of all the 6 fetuses studied was capable of splitting estrone sulphate vigorously. One per cent homogenate split 50—80 per cent of the substrate during an incubation of 20 hours. No hydrolysis of steroid sulphates was observed in these few experiments in the kidney, adrenal gland, pancreas and spleen. DHAS was not split by any tissue in human fetus. Like the placenta of fullterm babies, the fetal placenta was able to split steroid sulphates in the 3 cases studied.

Human alimentary canal samples obtained at operations, various tissues of the human fetus, tissues of rats of various ages and human placentas were used as the source of the enzyme to study the hydrolysis of androsterone sulphate. Potential splitting was studied at different pH (5—9) levels by incubating AS with 10 per cent homogenate for 6—20 hours. No hydrolysis of AS was observed in any of the samples.

IV
DISCUSSION

There can be some uncertainty about using whole homogenate as the source for studying an enzyme. Several fractions can sometimes be described by the same enzyme name, and when this happens it is not known what is being measured. Against this, however, it is arguable that it has not been possible to isolate arylsulphatase in perfectly pure different fractions. NPS, used in the present work, is known as the substrate of fraction C which it splits. However, fraction A also has some affinity towards NPS (Dodgson, Spencer and Wynn 1956). Fraction B does not split NPS at all. The optimum reaction milieus of different fractions for the same substrate vary. The optima obtained and used in the present work for human fetus were identical with those obtained by Dodgson, Spencer and Wynn with pure fraction C (1956), for liver optimum pH 7.3 and optimum substrate concentration 0.008 M. The splitting ratio of NPS and nitrocatechol sulphate (corresponding approximately to fraction C and fraction A + B) in man is 1 : 0.4 in liver, 1 : 0.5 in pancreas, 1 : 1.6 in kidney, 1 : 3.4 in lung, 1 : 4.1 in brain, 1 : 2.2 in heart, 1 : 2.3 in large intestine and 1 : 1.7 in small intestine. It is possible that owing to its obvious more profuse presence especially in lungs and brains but also in kidney and intestine, there may have been a small amount of fraction A in the analysis. It can be concluded that in the present work C was at least the predominant fraction, if not the sole fraction determined from the human fetus.

The optimum results for rat tissues are also interesting. Dodgson, Spencer and Thomas (1955) obtained 7.0 as the optimum pH for fraction C and 5.8 for fraction A. The attention is drawn by the fact that different optima for two different tissues, liver and kidney, 7.0 for the former and 5.8 for the latter were obtained in the present work. There might thus also be great differences in the localisation of the fractions in the various organs, as has earlier been shown to be the case in part in human tissues.

Table XVI. Arylsulphatase of acetone-dried adult tissues according to Dodgson, Spencer and Wynn (1956) compared with that of fetal crude homogenates in fairly similar incubation conditions in the present work. Results is calculated as μg liberated p-nitrophenol / gr wet wt. / hr.

Tissue	Dodgson	Present author
liver	35550	12580
pancreas	16600	1860
kidney	13700	1240
spleen	3500	45
small intestine	2500	5970 (jejunum)
lung	1150	400
large intestine	950	4240
brain	510	135
heart	100	70

The use of fresh rat liver homogenate has been warned against, not only for the lack of a more accurate definition of the fraction but also because of the metabolisation of p-nitrophenol. Obviously, however, this drawback did not affect the present work. The rat strain may be to blame for the failure to metabolise p-nitrophenol. Reference may be made here to earlier reports that major metabolisation was not observed in the other tissues in either mice or rats (DODGSON and SPENCER 1953a).

There are no reports of systematic observations of the stability of sulphatase. It generally remains stable for several months at —15°C. The present results confirmed that it keeps relatively well at —15°C, for months even. Assessed against the heat inactivation of enzymes in general, it can perhaps be said that sulphatases rank somewhere in the middle as regards stability.

The localisation of arylsulphatase in the different organs in human fetus revealed a fair similarity with the findings of DODGSON, SPENCER and WYNN (1956) in their study of enzyme activity in adult man. For comparison of the activity, table XVI shows the results of their study and the present results, both calculated as μg of liberated p-nitrophenol per g of wet weight tissue; the pH and the substrate concentration were the same in practice.

The results are naturally not fully comparable. There is no doubt that DODGSON, SPENCER and WYNN measured fraction C in their investigation, while there was no full certainty of the fractions in the present work. The substrate was the same, also the buffer and its pH. Another differ-

ence was the age of the material: DODGSON, SPENCER and WYNN used material from adults who had been dead for some time, the present author used fresh fetuses.

It is to be noted, however, that in both adults and fetuses liver is the organ that contains the most arylsulphatase. DODGSON found profuse enzyme in the pancreas and the kidney, and the same observation was made in the present investigation. However, with this method the activity in these fetal tissues was only 1/10 of its level in an adult, while in the liver it was 1/3 of the latter. The comparison is not really valid, of course, since among other things the homogenate percentage (0.2) used in the present work was fairly low for liver and the homogenate percentage for pancreas and kidney was 1. In fetal intestine, however, arylsulphatase was most profuse in the region of the small intestine, as was the case in adults. Taken as a whole, the intestine is much richer in enzyme in the fetus than after the termination of extrauterine life. This may be attributed in part to meconium, one important component of which derives from arylsulphatase-rich liver, in part to the paucity of enzyme-poor connective tissue. Arylsulphatase is in fact localised in the intestines in the mucosa (PULKKINEN, unpublished observation), as it usually is in the epithelial cells (RUTENBURG, COHEN and SELIGMAN 1952). On the other hand, when all the meconium had been washed away carefully with water no great fall in enzyme activity ensued in the present work, suggesting that the fetal intestine, unlike the adult intestine is rich in arylsulphatase, richer than the pancreas or the kidney.

The greatest difference seen in the comparative table, however, is in the values for the spleen. The values for human fetus are at the lower limit of the method, while values have been recorded for adults which are c. 1/4 of the activity of the kidney and in the size class of the intestines. It might be thought that there is an inhibitory factor in the fetal spleen, but incubation of splenic homogenate with hepatic homogenate produced no fall in the activity of the latter. On the other hand, the function of the spleen in the fetal period as a blood-forming organ is known to differ from that in the adult. The difference observed might be a manifestation of this.

The lung, brain and heart are otherwise engaged in both the investigations in the same order of activity. The concentration in the lung in both investigations was 2—3 times greater than in the brain. The cardiac muscle was the organ poorest in arylsulphatase.

Viewing the results against those arrived at by HUGGINS and SMITH (1947), no quantitative comparison proper is possible as these workers used a pH and substrate concentration considerably lower than the optimum. They also observed that activity in the adult was most profuse in the liver. The spleen, too, was also fairly rich in arylsulphatase. Worthy of special mention is the fact that they studied at the same time the cardiac and skeletal muscle and found the former to have more arylsulphatase, as in the present work.

The literature also has a mention of an attempt to determine the arylsulphatase of bone marrow (FOLIS 1951). Hardly any activity was observed and none at all was demonstrated in the cartilage. The same observation applied to the fetus.

The main period of fetal development in question in the present investigation was the organogenetic period. In all the tissues with activity of measurable concentration, the tendency was a rising one with the development of the fetus, and this applied to both wet weight and tissue nitrogen values. The failure to establish a statistically significant increase in only two tissues may have been due to the shortness of the period of observation for human fetuses. This arose from the natural limitations governing the performance of *sectio minor*.

A very pronounced regression was established between fetal length and arylsulphatase concentration in the kidney and the lung. It is difficult to say why this is so just in these tissues. Considered methodologically, these two organs contain a larger amount of fraction A than the liver. The lung is embryologically tissue which must develop to full functional readiness before birth but which does not function during fetal life. Nearly the same can be said of the kidney for metabolism does in fact take place via the placenta. Metanephrogenic tissue diminishes throughout fetal development and nephrons are formed all the time (STARCK 1955). The difference e.g. from the development of the liver is distinct in this respect since, for reasons of metabolism, the liver must in many respects be capable of function in the fetal period already.

The arylsulphatase activity of the kidney and the lungs rises almost equally sharply and relatively linearly, and the shape of the curve does not help to illustrate the point any better. As arylsulphatase in the kidney has been localised in the nephrons (RUTENBURG, COHEN and SELIGMAN 1952), linear increase concurs with the embrylogical finding that the nephrons likewise increase relatively evenly.

Compared with other enzymes, the results obtained for the change in arylsulphatase concentration are not always analogous. SAVOLA (1957) studied the β-glucuronidase content of human fetuses and found that it rose fairly sharply also in liver. There are in fact several reports in the literature that β-glucuronidase and arylsulphatase do not run a parallel course (DODGSON, LEWIS and SPENCER 1953, HAYASHI et al. 1955a and b, HAYASHI et al. 1957, SELIGMAN, NACHLAS and COHEN 1950).

The arylsulphatase of the placenta has been studied earlier (BIANCHI 1955a, BIANCHI and VALLI 1955). Their findings for the quantity of the enzyme are 1/10 of the values obtained in the present study; the reaction milieus were different. BIANCHI washed the placentas with physiological saline (activator) but made his measurements at a more acid pH (inactivation). He attached great importance to arylsulphatase in placenta and said that arylsulphatase causes changes in the metabolism of hypertensive amines because the arylsulphatase content is lower in toxemia. The pre-eclamptic placentas included in the present series do not support this view, but the materials of both investigations were too small for proper evaluation of the question.

The water content of the earlier fetal period is great. This makes it understandable that a significant regression was established between fetal length and enzyme activity of corresponding placenta in the wet weight analysis. No increase occurred in the results per tissue nitrogen during the observation period. Clearly, the intrapartum placenta possesses a considerably higher arylsulphatase content per weight unit although not per unit of tissue nitrogen. Without forgetting the role played by arylsulphatase in the metabolism of phenol sulphates, mention might be made in this connection of the free estrogens circulating in the maternal blood at birth (REYNOLDS 1949). For example, estrone sulphate is split into free estrogen by arylsulphatase. On the other hand, the clearance of sulphates in the kidney is smaller than that of glucuronates (KELLIC and SMITH 1957). The sulphate form is probably more common in the blood, glucuronate is more a secretory form. The placenta (also the fetus itself) possesses higher arylsulphatase concentrations during birth, i.e. a greater capacity to split estrogen sulphates. This is surely not the sole explanation of the phenomenon, but it may be a part factor. In addition to the rise in the splitting capacity in the placenta per gramme, the total weight also rises.

Following the arylsulphatase concentrations in rat material during

intrauterine life gives a clear idea of the real increase in the enzyme towards birth. The phenomenon can be regarded as one of many that prepare the organism for the more mature extrauterine life. Many of the natural substrates possessed by arylsulphatase become important for the individual once extrauterine life has started, the time when the biological factor studied here is generally ripe to assume its tasks.

The most distinctive detail in the organ distribution of arylsulphatase in human and rat fetus is the almost complete absence of the enzyme in the intestines of the rat.

No statistically significant increase with age was established in human fetal liver during the observation period but there was a rising tendency. Rat fetuses displayed a distinct rise ($r = 0.525$ and 0.392 for the U_w and U_N values). It is possible that a corresponding development occurs during the intrauterine evolution of man although it was not observed in the present work owing to natural limitations.

In kidney, again, a very highly significant increase in arylsulphatase was established in both parts of the material. The relatively very close r values (for human fetus r was 0.713 and for rat fetus 0.619 in the wet weight analysis) and the sharpness of the rise seem to be a general feature. The purpose for which the kidney secreting arylsulphates via glomerular filtration (Kellic and Smith 1957) needs the arylsulphatase rests on mere speculation. The function theory introduced by Suzuki, Takahashi and Egami (1957) may be mentioned again in this connection. As arylsulphatase is able to transfer the sulphate from an arylsulphate to another aryl group, the kidney may be able to affect this secretion and make it selective.

Comparison of the arylsulphatase concentration of newborn rat with that of large fetuses close to birth gave values of the same magnitude. Hence no major changes in the enzyme content occur around birth; the development that has begun goes on fairly evenly. The change to extrauterine life has no especial influence on the content of the enzyme.

During the development of rat to sexual maturity the enzyme concentrations increase in both liver and kidney. It is obvious that age affects the concentrations later, too, and that the level does not remain stable throughout life. The maximum age (c. 30 years) of the arylsulphatase of human urine established by Ammon and Ney (1957), for example, speaks in favour of this assumption.

A sex difference was established in the enzyme of rats (Dodgson, Spencer and Thomas 1953b). It was demonstrated in rats of the Medical

Research Council. The analysis was confined to the wet weight of liver enzyme with p-acetylphenyl sulphate as the substrate. The present investigation established a corresponding significant difference in adults, though relatively it was much smaller. This difference may be due e.g. to the rat strain used. In the kidney the difference is the reverse. Such a phenomenon is not unknown enzymologically. For instance, the β-glucuronidase concentration in the kidney increases when androgen is used, but this does not occur in the liver (FISHMAN and FARMELANT 1953). It has been demonstrated that estrone, on the other hand, increases the liver β-glucuronidase of ovariectomised mouse (KERR, CAMPBELL and LEVVY 1949). As is only natural, no sex difference was established in this study in the sexually immature rats of the strain used (i.e. rats aged 1 month). The difference emerged only with sexual maturity. However, the phenomenon was not observed in the nitrogen values and it is therefore possible to speak only of a certain tendency.

The β-glucuronidase concentration of rat liver and serum is increased in pregnancy (BERNARD and ODELL 1950). A direct consequence of this is that conjugates can be freed most effectively during pregnancy. The ratio between 17-hydroxyketosteroids and its conjugates is changed during pregnancy, free steroids become relatively much more numerous (PEKKARINEN and RAURAMO 1960). In addition to lowered conjugation capacity, this may be due also to the possibility that conjugates are hydrolysed more rapidly than usual in this condition. A source of great potential increase in this respect is the ability of the placenta to split steroid conjugates extremely efficiently.

In addition to the pronounced arylsulphatase activity established and its increase with the maturing of the placenta, the placenta also contains profuse β-glucuronidase (FISHMAN 1955) which splits the corresponding conjugates of glucuronic acid.

The absence of specific steroid sulphatase in the fetus and the newborn can be regarded as an indication of their insufficient steroid metabolism. It was seen that rat fetuses did not hydrolyse estrone sulphate although they possessed arylsulphatase and this sulphate should be split by the same enzyme. The method used, however, makes it uncertain which fraction is being measured with the crude homogenate. Secondly, there are no detailed studies to clarify which fraction splits estrone sulphate. The matter calls for further investigations. It has been demonstrated, nevertheless, that human fetal liver splits estrone sulphate as expected. It is difficult to assess the importance of this obser-

vation for the steroid balance during pregnancy. Free steroids in general are regarded as having a stronger influence than conjugated steroids (BISHOP, RICHARDS and PERRY 1951). Many estrogen sulphates are substrates of unspecific arylsulphatase and the fetus thus "produces" free estrogens. The fetus does not interfere with the 5 α- and Δ^5-steroids of 3 β-sulphates since there is no specific steroid sulphatase. The free steroid form of DHAS used and common in nature, i.e. dehydroepiandrosterone, is probably of no great importance for the pregnant organism.

Specific steroid sulphatase also appears soon after birth. The quantity found in a rat of no more than 1 month of age is almost the same as in an adult animal. There is no absolute certainty whether it increases further from this level towards adulthood. At least the tendency could be seen. A part of the natural substrates of specific steroid sulphatase are sex hormones, but there are also fairly numerous conjugated secreta of the adrenal gland. It is thus understandable that a month-old rat can have such a marked steroid sulphatase content in liver.

No sex differences were established for this enzyme. The explanation again may be the strain used; the limitations of the method must also be considered.

The splitting of estrone sulphate increased statistically significantly throughout the period studied. The result is analogous with the finding for NPS. With the exception of substrates for arylsulphatase in estrone sulphate this can be regarded as natural. Estrogen production increases and at the same time there is an increase in the enzyme which regulates the balance between free and conjugated hormones and thus, via their physiological effect of different value, the changes in the organism displayed by these hormones.

The relatively high concentrations of steroid sulphatase in the placenta cannot be without biological significance. The specificity of the enzyme makes it interesting in this respect. The purpose probably is to be able to liberate during pregnancy an increasing number of steroid conjugates as free hormones. The presence in the placenta of an enzyme with many kinds of substrates (such as arylsulphatase) cannot be considered binding proof of its effect on steroid metabolism. But as far as steroid sulphatase is concerned there is no room for interpretation in this sense — it affects steroid sulphates only. In addition, some other steroid sulphates are also readily hydrolysed, e.g. the estrone sulphate used here.

To return to 3 α-steroid sulphates, it must be remembered first that the form is not physiological (Roy 1956c); these steroids tend to become glucuronates, the form in which they are secreted (Staib, Teller and Scharf 1960). Androsterone sulphate did not in fact split the tissues of any species in the different experimental conditions. This may also be regarded as proof of the biological task of the sulphatase which really split conjugates and which have their own substrates in nature. The placenta, no more than any other of the tissues studied, was unable to produce androsterone in free form.

No differences were observed in the enzyme concentrations of the different sides of the placenta. Macroscopic cutting probably gives fairly similar tissue. On the other hand, the placenta is composed almost entirely of fetal material (Starck 1955).

The literature unfortunately contributes nothing to the histochemical localisation of sulphatases in the placenta. It is generally assumed that steroids are localised in syncytiotrophoblasts (Wislocki and Bennett 1943).

It is noteworthy that the placenta of fetuses contains less arylsulphatase. A notable histological feature is the disappearance of Langhans' cells by the 4th—5th month of pregnancy (Starck 1955). However, as the enzyme concentrations are higher in intrapartum placenta than in the 2nd—5th month of pregnancy it might be assumed that, like the steroids, sulphatases are localised in syncytiotrophoblasts.

Does steroid sulphate split *in vivo*? It has been observed that if a female rat is given ^{35}S sodium estrone sulphate intravenously, subcutaneous or by tube into the stomach, 75 per cent of the radioactive ^{35}S estrogen is found within 36 hours in the urine and only in free form (Hanahan and Everett 1950). Fifteen per cent is excreted into the bile. ^{35}S sodium estrone sulphate administered to a pregnant rat did not split when it penetrated the placenta on the fetal side (Hanahan, Everett and Davis 1949). The result is fully analogous with that obtained in the present investigation with the *in vitro* analysis of liver homogenates of rat fetus. It should be pointed out in this connection that human fetal liver was capable of hydrolysing estrone sulphate. The missing splitting capacity of the substrate was thus obviously specific for the strain.

The synthesis has been known long *in vivo*. Among the most recent studies is that of Staib, Teller and Scharf, 1960, who established that 3β-steroids not unlike dehydroepiandrosterone were excreted as

sulphate and 3α-steroids as glucuronate. The adrenogenital syndrome of puberty, which also displayed adrenal hyperplasia, was an exception. In this fairly abundant androsterone sulphate was excreted which was not normally observable even when the patient was given 100 mg of androsterone per os. In conditions like these, where androsterone sulphate synthesis is possible, its cleavage might also be established with biological material, which cannot be done in a normal organism.

The natural function of sulphatases is to split conjugates *in vivo*. Their activity results first in fewer conjugates and secondly in more numerous liberated phenols, steroids, etc. As regards the first alternative, relatively little is known of the biological tasks of sulphate conjugates. If the other component is the poisonous phenol, it must be merely a kind of excretion that is involved, a mode of detoxication. An example of the causal relationship that may be involved is the correlation between certain carcinogenic amines, their sulphate conjugates and sulphatase in patients with cancer of urinary bladder. The normal metabolites of tryptophan, i.e. 3-hydroxyanthraniline acid and 3-hydroxy-kynurenine, are carcinogenic when implanted in mouse bladder (Bonser et al. 1953). They occur chiefly as conjugates in the urine of healthy persons (Boyland and Williams 1955), whereas patients with cancer of the urinary bladder have a relatively greater number of free carcinogens. It is known that carcinogens are generally conjugated e.g. into sulphates, in which form they lose their carcinogenicity. Moreover, sulphatases is generally unable to split these conjugates (Boyland et al. 1956). It has been established, however, that the urine of patients with cancer of the urinary bladder contains an abnormally high amount of sulphatase (Boyland, Wallace and Williams 1954, Dzialoszynski 1957), and consequently there may be a causal relationship.

The old argument that sulphate conjugates are more inactive than free sulphates is only partly true. The contrary has been suggested and supported by evidence. For instance, the sulphate conjugates of di-ethylstilbestrol, estradiol and estrone inhibit kynurenine transaminase in very low concentrations, while free estrogens are ineffective even in saturated solutions (Mason and Gullekson 1960). Hence, if sulphatase is used to split steroid conjugates *in vivo* stimulation may occur in some function, but in the light of the example above it is also possible that retardation may occur.

It is difficult to assess the *in vivo* effect from *in vitro* experiments.

The amount of phosphate contained in the kidney physiologically, e.g. in intracellular fluid, is capable of considerable inhibition of aryl-sulphatase A and B.

These few aspects perhaps illustrate the rôle of sulphatase. The results obtained in the present work support the view that the function of sulphatases must be comparatively wide. The localisation in so many organs, its increase as the organism grows and the high concentrations in the steroid-producing placenta are such facts.

V

SUMMARY

(1) Arylsulphatase was determined by modification of the p-nitrophenol sulphate method and the splitting of steroid sulphates was studied according to Roy by spectrophotometric measurement of the unsplit steroid sulphate as methylene blue salt extracted with chloroform.

The pH optimum of arylsulphatase was 7.3 in man, 7.0 in rat liver and 5.8 in rat kidney. The optimum substrate concentration in human fetus was 0.008 M for p-nitrophenyl sulphate. No metabolisation of p-nitrophenol was established in vitro in these experimental conditions in rat or human fetal tissues. The recovery of protein-containing material was good when zinc hydroxide was used for precipitation. The maximum absorption of p-nitrophenol was at 402 mμ. The enzymatic reaction was relatively linear in the period studied.

Arylsulphatase kept well for one month at −15° C. At +4° C there was a relatively rapid decrease in enzyme activity and after 24 hours at +20° C only a part of the activity remained.

(2) Dehydroepiandrosterone sulphate, estrone sulphate and androsterone sulphate were employed as steroid sulphates. The optimum pH of human placenta and rat liver was approximately 7.8 in TRIS buffer when the substrate concentration was 0.2 mM.

The absorption maximum of the complex of steroid sulphate-methylene blue extracted with chloroform was 663 mμ in an alcoholic solution. The hydrolysis was relatively linear to the reaction time with an incubation of 3—6 hours. The maximum substrate hydrolysing degree was 50 per cent.

(3) The human fetus series was collected from *sectio minor* operations for legal abortions. It consisted of 73 fetuses with a crown-heel length of 0.5—33 cm. A total of 811 duplicate determinations of arylsulphatase was made on this part of the material.

Relatively high arylsulphatase activity was established in some tissues of the human fetus. The results were expressed per μg of liberated p-nitrophenol/100 mg wet wt./hr (U_w) and per mgN/hr (U_N). The liver showed the highest enzyme concentration (1260 ± 70 U_w and 770 ± 50 U_N), followed by the ileum (675 ± 50 U_w and 410 ± 36 U_N), jejunum, duodenum, colon, pancreas (186 ± 17 U_w and 134 ± 12.1 U_N), kidney (124 ± 8.8 U_w and 118 ± 8.1 U_N), lungs (40.3 ± 3.6 U_w and and 47 ± 3.7 U_N), brain (13.5 ± 1.24 U_w and 17.7 ± 1.48 U_N), genitals, salivary glands and adrenal gland. The amounts in the heart, thyroid gland, spleen and skeletal muscle were at the lower limits of the method and no activity at all was established in bone marrow and bone.

A statistically significant increase in arylsulphatase activity was established in human kidney and lung during the period of fetal growth observed.

(4) Arylsulphatase activity was established in 55 fetal placentas. It was 191 ± 11.2 U_w and 183 ± 14.1 U_N in the samples taken from the fetal side and 171 ± 12.1 U_w and 156 ± 15.6 U_N in those taken from the maternal side. There was no significant difference between the two sides.

In the 29 placentas from spontaneous deliveries the activity was 218 ± 17 U_w and 86.6 ± 6.90 U_N in the samples from the fetal side and 241 ± 15.1 U_w and 104 ± 5.2 U_N in those from the maternal side. The placenta showed a significantly greater amount of arylsulphatase per weight unit during delivery than in the fetal period. The reverse was true when measured in terms of tissue nitrogen.

(5) In rat, arylsulphatase activity occurs at least in the liver and kidney during intrauterine development. Analysis of the tissues of 65 fetuses revealed a significant increase in the activity of both these organs with fetal growth, and this is true even for the results calculated per tissue nitrogen which excluded the water content. The mean concentration in the liver in fetuses of different ages was 16.0 ± 1.12 U_w and 9.7 ± 0.27 U_N and in the kidney 12.9 ± 0.97 U_w and 7.6 ± 1.11 U_N.

The alimentary canal of rat fetus contained very little arylsulphatase, 6.3 ± 0.31 U_w and 4.6 ± 0.25 U_N; the difference from the human fetus was distinct.

The increase in activity continued post partum. The liver, kidney and intestine of 46 newborn, 48 month-old and 54 adult ($3\frac{1}{2}$ months

old) rats were analysed for arylsulphatase. Intestinal tissue gave small enzyme activity values. The hepatic arylsulphatase concentration grew from 26.1 ± 1.75 U_w at birth to 109 ± 4.4 U_w in adults and 11.9 ± 0.87 U_N to 38.3 ± 1.83 U_N. The increase was significant also between newborn and month-old animals. The corresponding values for the kidney were 32.5 ± 2.2 and 81.0 ± 2.56 U_w, 17.7 ± 1.93 and 28.8 ± 1.41 U_N.

No definite sex difference was established in the rat strain used. The mean for male rats was greater than the mean for the females, and in the liver the difference was statistically significant per wet weight. The kidney gave the opposite result.

(6) Six human fetuses and 103 rats were used to study the hydrolysis of steroid sulphates. The tissues did not hydrolyse androsterone sulphate even when the reaction conditions were modified.

Steroid sulphatase was not observed in the eigth tissues of human fetus studied, nor in the liver of rat fetus or newborn rat. The liver of month-old rat, on the other hand, has almost the same level of enzyme activity as an adult rat. No sex difference was observed in the rat strain used.

Estrone sulphate was not hydrolysed in rat fetal liver, but the liver of human fetus was able to split it easily. Newborn rat possesses the splitting ability, a capacity which grows in the animal as it develops.

(7) Twenty nine placentas from spontaneous deliveries and six fetal placentas were studied for their ability to hydrolyse steroid sulphates. Human placenta in all its phases contains profuse steroid sulphatase and splits estrone sulphate readily. No significant difference was established between the samples from the fetal and those from the the maternal side.

IV

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